STUDIES ON SOLUBLE AND IMMOBILIZED PEROXIDASES AND THEIR APPLICATIONS IN THE DECOLORIZATION OF AROMATIC DYES

SUMMARY

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

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IN

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BY

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Summary

Enzymes have a great appeal in chemical processes as 'green chemistry' reagents that will allow future sustainable developments. In this sense, enzyme technology has become a multidisciplinary field with applications in diverse processes. Peroxidases in particular have been extensively studied and show many attractive properties for biocatalysis such as wide specificity, high stability in solution and easy accessibility from plant materials. Bitter gourd and turnip peroxidases have generated great interest due to its different activity and stability profile compared to its widely used counterpart, horseradish peroxidase. Immobilization is an efficient way to prevent inactivation and extend enzyme half-life, besides, immobilization procedures can increase structural rigidity thus improving pH, temperature and organic solvent tolerance.

In the present study, an effort has been made to immobilize peroxidases directly from ammonium sulphate precipitated proteins of bitter gourd on an anion exchanger, DEAE cellulose. The activated DEAE cellulose was quite effective in high yield immobilization of peroxidases from bitter gourd and it bound peroxidase activity 590 U g^{-1} of the matrix. Bitter gourd peroxidase immobilized on this anion exchanger showed very high effectiveness factor 'η' as 0.95. BGP could bind very strongly to the DEAE cellulose, as it did not detach even in the presence of 0.5 M NaCl. The incubation of immobilized enzyme with 0.1 M NaCl for 24 h resulted in a loss of only 13% of the initial activity. Immobilized bitter gourd peroxidase preparation was more stable to denaturation induced by pH, heat, urea, proteolytic enzyme, detergents; Surf Excel and Rin Powder, Triton X-100 and water miscible organic solvents; dioxane, dimethyl sulphoxide and *n*-propanol. Peroxidase adsorbed on the matrix exhibited very high resistance to proteolysis mediated by the trypsin treatment. DEAE cellulose bound bitter gourd peroxidase lost 45% of its initial activity after treatment with 2.5 mg mL^{-1} of trypsin for 1 h at 37 °C while the soluble enzyme lost nearly 65% of the initial activity under similar incubation conditions.

Turnip peroxidase is also a very attractive and cost-effective candidate for bioremediation because it is obtained from turnip roots which are easily available in northern part of India. In order to keep costs down, especially with respect to traditional chemical processes, several attempts have been made to find an enzymatic method that could be easily applied on an industrial scale. Concanavalin A is finding increasing applications as a useful ligand in glycoenzyme immobilization. Concanavalin A adsorbed cellulose has been employed for the simultaneous purification and immobilization of glycoenzymes directly from ammonium sulphate fractionated proteins of turnip. The bioaffinity support was prepared simply by incubating cellulose powder with jack bean extract at 4 °C. Cellulose powder adsorbed 30 mg concanavalin A g^{-1} of the matrix. The obtained bioaffinity support was quite effective in high yield immobilization of peroxidase from turnip and it retained 672 U g^{-1} of the matrix. Turnip peroxidase immobilized on concanavalin A-cellulose support retained 80% of the initial activity. Turnip peroxidase bound very strongly to Con A-cellulose support as it did not detach even in the presence of 0.8 M NaCl. Immobilized turnip peroxidase preparation was compared with its soluble counterpart for the stability against various forms of chemical and physical parameters. Immobilized turnip peroxidase preparation was quite resistant against the denaturation mediated by pH, heat, urea, guanidinium-HCl, detergents; Surf Excel, cetyltrimethylammonium bromide, Triton X-100, Tween 20 and water-miscible organic solvents; dimethyl formamide, dioxane and *n*-propanol. Low concentrations of detergents like Surf Excel and cetyltrimethylammonium bromide enhanced the activity of soluble and immobilized turnip peroxidase. The activity of soluble and immobilized turnip peroxidase was also enhanced in the presence of lower concentrations of non-ionic detergents (0.2-1.0%, v/v) like Triton X-100, Tween 20. This enhancement in enzyme activity of immobilized turnip peroxidase was remarkably very high.

The role of partially purified turnip peroxidase for the treatment of acid dyes has been investigated. Turnip peroxidase was fractionated from the buffer extract by 20-80% $(NH_4)_2SO_4$ precipitation. Peroxidases from turnip roots were highly effective in decolorizing acid dyes having wide spectrum chemical groups. Dye solutions, containing 40-170 mg L^{-1}, were treated by turnip peroxidases (specific activity of 122.0 U mg^{-1} proteins). The parameters such as the effect of enzyme concentration, time, pH and temperature were standardized for the decolorization of acid dyes. Turnip peroxidase was able to decolorize most of the acid dyes in the presence of 2.0 mM 1-hydroxybenzotriazole. Increasing concentration of enzyme and time in the absence of 1-hydroxybenzotriazole did not influence dye decolorization. The rate of decolorization was significantly enhanced when 1-hydroxybenzotriazole was added to the decolorizing solutions. In initial first hour, most of the dyes were maximally decolorized. The decolorization of all the used dyes was maximum at pH 5.0 and 40 °C. In order to prove the compatibility of enzyme in the treatment of industrial effluents, we have investigated the treatment of mixtures of dyes with partially purified turnip peroxidase. Complex mixtures of dyes were significantly decolorized when treated with enzyme in the presence of 1-hydroxybenzotriazole (2.0 mM). Phytotoxicity test based on *Allium cepa* root growth inhibition has shown that majority of the turnip peroxidase-treated dye product were less toxic than their parent dye. Kinetic parameters of the turnip peroxidase with various dyes showed that this enzyme has highest affinity for Acid Yellow 42. The polluted wastewater contaminated with single dye or mixtures of dyes were treated with enzyme and it resulted in a remarkable loss of total organic carbon content. This study demonstrates that the peroxidase/mediator system was an effective biocatalyst for the treatment of effluents coming out from textile, dye manufacturing, dyeing and printing industries or complex mixtures of dyes.

Decolorization and decontamination of two textile carpet industrial effluents was carried out by using Con A-cellulose bound turnip and tomato

peroxidases. Both effluents were recalcitrant to the action of these plant peroxidases. However, the decolorization of effluents was enhanced in the presence of 2.0 mM HOBT. Industrial effluents; textile carpet effluent red and textile carpet effluent blue were decolorized 75% and 80% by soluble turnip peroxidase (0.423 U mL^{-1}) and 69% and 59% by soluble tomato peroxidase (0.705 U mL^{-1}), respectively. After 15 min, textile carpet effluent red and textile carpet effluent blue were decolorized 65% and 56% by turnip peroxidase; however, these effluents were decolorized 63% and 51% by tomato peroxidase. Both effluents were maximally decolorized at pH 5.0 by soluble and immobilized turnip peroxidase whereas the maximum decolorization by soluble and immobilized tomato peroxidase was found at pH 6.0. However, it was observed that both effluents were decolorized maximally at 40 °C by soluble and immobilized peroxidases. The immobilized turnip peroxidase treated effluent exhibited significant loss of total organic carbon content from the solution. These observations suggested that major toxic compounds get easily removed out of the turnip and tomato peroxidase treated samples. Immobilized peroxidase preparations have a marginal edge over the soluble enzyme in decolorizing the textile effluents. The absorption spectra of treated and untreated textile effluent exhibited a marked difference in the absorbance in the visible region. The effluent decolorizing reusability was continuously decreased up to their 8^{th} repeated use. Immobilized turnip peroxidase showed over 40% decolorization even after its 8^{th} use. This study showed that turnip peroxidase was more efficient than tomato peroxidase in the decolorization and degradation of textile effluents.

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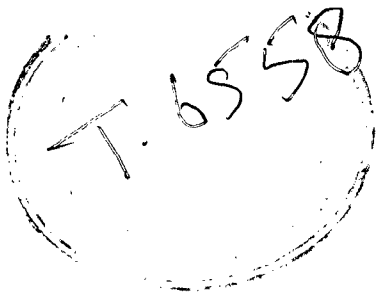

To
My Grand Parents
with
Love and Affection

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Certificate

This is to certify that the thesis entitled ***"Studies on soluble and immobilized peroxidases and their applications in the decolorization of aromatic dyes"*** *being submitted by* ***Yasha Kulshrestha*** *to Aligarh Muslim University, Aligarh for the award of the degree of* ***Doctor of Philosophy in Biochemistry*** *is a record of bonafide research work carried out by her. Yasha Kulshrestha has worked under my guidance and supervision and has fulfilled the requirements for the submission of this thesis, which to my knowledge has reached the requisite standard.*

The results contained in this dissertation have not been submitted in part or in full to any other University or Institute for the award of any degree.

Q. Husain

Prof. Qayyum Husain

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In the name of god, the Almighty, who guided me and showed me the right path of life, enabling me to achieve this target.

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(Yasha Kulshrestha)

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LIST OF ABBREVIATIONS

2 Cl-1,4-DMB	-	2-chloro-1,4-dimethoxybenzene
AP	-	Aminopyrine
ARP	-	*Arthromyces ramosus* peroxidase
BGP	-	Bitter gourd peroxidase
CIP	-	*Coprinus cinereus* peroxidase
COG	-	Crocein Orange G
Con A	-	Concanavalin A
CP	-	Catalase peroxidase
CPZ	-	Chlorpromazine
CTAB	-	Cetyltrimethylammonium bromide
DAT	-	Diaminotoluene
DDT	-	1,1,1,-trichloro-2,2-bis (4-chlorophenyl) ethane
BTEX	-	Benzene, toluene, ethylbenzene, and xylene
DEAE	-	Diethyl aminoethyl
DMF	-	Dimethyl formamide
DMSO	-	Dimethyl sulphoxide
DNR	-	2,4-dinitrosoresorcinol
DyP	-	Dye-decolorizing peroxidase
eDyP	-	Expressed dye-decolorizing peroxidase
GS*	-	Glutathionyl free radical
HOBT	-	1-hydroxybenzotriazole
HPLC	-	High performance liquid chromatography
HRP	-	Horseradish peroxidase
IBGP	-	Immobilized bitter gourd peroxidase
IEA	-	Isoeugenyl acetate
ITMP	-	Immobilized tomato peroxidase
ITP	-	Immobilized turnip peroxidase
Lac	-	Laccase
LiP	-	Lignin peroxidase
LMEs	-	Lignin modifying enzymes
MMA	-	4-methoxy mandelic acid
Mn LiP	-	Manganese-lignin peroxidase

MnP	-	Manganese peroxidase
MP-11	-	Microperoxidase-11
M_r	-	Molecular weight
NBT	-	Nitroblue tetrazolium
NEPT	-	N-ethyl phenothiazine
NsP	-	Non-specific peroxidase
PAH	-	Polycyclic aromatic hydrocarbon
PCB	-	Polychlorinated biphenyl
PCP	-	Pentachlorophenol
PEG	-	Polyethylene glycol
POX	-	Peroxidase
RBBR	-	Remazol Brilliant Blue R
SBGP	-	Soluble bitter gourd peroxidase
SBP	-	Soybean peroxidase
SP	-	Sulfonaphthalein
STMP	-	Soluble tomato peroxidase
STP	-	Soluble turnip peroxidase
TCEB	-	Textile carpet effluent blue
TCER	-	Textile carpet effluent red
TCP	-	Trichlorophenol
TMB	-	3,3'5,5'-tetramethylbenzine
TMP	-	Tomato peroxidase
TNT	-	2,4,6-trinitrotoluene
TOC	-	Total organic carbon
TP	-	Turnip peroxidase
UKP	-	Hard wood kraft pulp
UV	-	Ultra violet
VA	-	Veratryl alcohol
VLA	-	Violuric acid
WRF	-	White rot fungi

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CHAPTER-I

Review of Literature

1.1. ORGANOPOLLUTANTS AS ENVIRONMENTAL CONCERN

One of the major environmental problems facing the world today is the contamination of soil, water and air by toxic chemicals. Eighty billion pounds of hazardous organopollutants are produced annually in the United States and only 10% of these are disposed of safely (Reddy and Mathew, 2001). There are several classes of chemicals that have been targeted by the USEPA as priority pollutants due to their toxic effects on the environment and human health. Certain Hazardous compounds, such as polycyclic aromatic hydrocarbons (PAH), pentachlorophenol (PCP), polychlorinated biphenyl (PCB), 1,1,1,-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), benzene, toluene, ethylbenzene, and xylene (BTEX), and 2,4,6-trinitrotoluene (TNT) are persistent in the environment and are known to have carcinogenic and/or mutagenic effects. PAH are pollutants produced via natural and anthropogenic sources, generated during the incomplete combustion of solid and liquid fuels or derived from industrial activities. These compounds are hydrophobic with low water solubility, thus they are easily adsorbed onto organic matter as soils and sediments. Besides their recalcitrant behavior greatly hampers their naturally biological degradation (Cerniglia, 1992; Shuttleworth and Cerniglia, 1995). PCBs were used extensively until 1993 in dielectric and hydraulic fluid, flame retardants, plastisizers, solvent extenders, textiles and printing (Reddy and Mathew, 2001). Estimates of total production of this volatile, bioaccumulative toxin range from 1.3-2.0 million tons worldwide (Breivik et al., 2004). Organochlorines are persistent in the environment, and as a result of biomagnification through the food chain, several organochlorine linked toxic effects/population declines at higher trophic levels have been recorded (Alloway and Ayres, 1993).

DDT is an organochloride insecticide that was banned in the United States over 30 years ago. This chemical is persistent in the environment, however biomagnifying through the food chain. Toxic effects and population declines at higher trophic levels due to DDT have been recorded, with some studies finding stable residues in air, water, soil, sediment, fish and birds more than 10 years after it was banned (Breivik et al., 2004). World production of TNT has been estimated at 900,000 kg annually (Harter, 1985). 2,4,6-trinitrotoluene is used as a major military explosive and can be an important

contaminant of soil and ground water at many sites of TNT production and storage. TNT is recalcitrant, toxic, and mutagenic to various organisms in the environment and, on the basis of animal studies, is also a possible human carcinogen (Honeycutt et al., 1996; Lewis et al., 1997). It is toxic to most organisms at 50 ppm but some sites have concentrations of 4,000-12,000 ppm (Boopathy, 2000).

Phenolic compounds contaminate many industrial wastewater streams, particularly those from the oil refining, polymer processing and fibre industries. They represent a potential danger to human health because almost all are toxic and many of them are known or suspected carcinogen (Sakurai et al., 2003). There are more than 8000 chemical products associated with the dyeing process listed in the color index, while over 1,00,000 commercially available dyes exist with over $7X10^5$ tons of dyestuff-produced annually (Zollinger, 1991). Interest in the pollution potential of dyes and dye intermediates has been primarily prompted by the concern over their putative toxicity and carcinogenicity, mainly due to the fact that many dyes are made from known carcinogens, such as benzidine, naphthalene and other aromatic compounds, all of which might be transformed as a result of microbial metabolism. Anthraquinone based dyes are the most resistant to degradation due to their fused aromatic structure, basic dyes have high brilliance and therefore higher color intensity, making them more difficult to decolorize, while metal-based complex dyes, such as chromium-based dyes can lead the release of chromium, which is carcinogenic in nature (Baughman and Perenich, 1988). All of these chemical compounds pose a significant threat to the health and vitality of the earth system and a sustainable method of detoxification is key.

1.2. CLASSICAL METHODS FOR THE TREATMENT OF ORGANO POLLUTANT AND THEIR LIMITATIONS

Conventional physical and chemical methods include membrane filtration, coagulation/flocculation, sorption and ion exchange, electrolysis, adsorption, advanced oxidation processes (chlorination, bleaching, ozonation, Fenton oxidation and photocatalytic oxidation) and chemical reduction (Vandevivere et al., 1998; Hao et al., 2000). Several factors, e.g. pollutant type, wastewater composition, dose and costs of

required chemicals, operational costs, environmental fate and handling costs of generated waste products determine the technical and economic feasibility of each single compound removal technique.

The physico-chemical techniques mentioned above have their own limitations. The use of one individual process may often not be sufficient to achieve complete mineralization of the compound. One major disadvantage of ion exchange is the high operation cost (Mishra and Tripathy, 1993). The high cost of chemicals for precipitation as well as for pH adjustments, problems associated with dewatering and disposing of generated sludge and high concentration of residual cation levels which remains in the supernatant are some of the limitations of coagulation and precipitation method (Anjaneyulu et al., 2005). Electrochemical methods are expensive due to large energy requirements and the limited lifetime of the electrodes (Vandevivere et al., 1998). The disadvantage of ozonation is its short half-life (typically being 20 min) demanding continuous application making it a cost intensive process (Xu and Lebrun, 1999). In Fenton oxidation, large volumes of waste sludge are generated at higher pH (Aplin and Waite, 2000). Drawbacks of the photocatalytic oxidation process are the relatively high costs and the occasional lack of effectiveness (Hao et al., 2000).

1.3. BIOLOGICAL METHODS OF TREATMENT

Recently, biological techniques based on microbial transformation or degradation of aromatic pollutants has attracted much attention in the treatment of wastewater. Many researchers have demonstrated partial or complete biodegradation of such chemicals by pure and mixed cultures of bacteria, fungi and algae (Field et al., 1995; Kirso and Irha, 1998; Pearce et al. 2003; Soares et al., 2006). An advantage of biological treatment over certain physico-chemical treatment methods is that over 70% of the organic material present that is measured by the COD test may be converted to biosolids (Anjaneyulu et al., 2005).

1.3.1. Bacterial biodegradation

Efforts to isolate bacterial cultures capable of degrading aromatic compounds started in the 1970s with reports of a *Bacillus subtilis* followed by *Pseudomonas* sp., *Escherichia coli* and Sulfate reducing bacteria (Anjaneyulu et al., 2005). Isolating such microorganisms proved to be a difficult task. Mixed bacterial cultures from a wide variety of habitats have been shown to transform the aromatic pollutants (Knapp and Newby, 1995; Field et al., 1995). Metabolic versatility of bacteria allows them to degrade aromatic pollutants (Eduardo, 2004).

1.3.2. Fungal biodegradation

White rot fungi (WRF) were first recognized as efficient degraders of organopollutants by Bumpus et al. (1985) and have been shown to transform recalcitrant environmental pollutants like PAHs and PCBs (Barr and Aust, 1992). Many species of WRF are capable of degrading a variety of xenobiotic compounds including PAHs, PCBs, insecticides, antibiotics and surfactants (Hatakka et al., 2001; Rigas et al., 2005; Kornillowicz-Kowalska et al., 2006; Soares et al., 2006) through the production of extracellular enzyme systems developed for the degradation of lignin (Andersson et al., 2001).

1.3.3. Algal biodegradation

Limited amount of work is available on the usage of algae for the degradation of aromatic pollutants (Jinqi and Houtian, 1992). Some species of algae were capable of utilizing a few aromatic pollutants as their sole source of carbon and nitrogen (Banat et al., 1996). The bioconcentration and transformation of a priority PAH, benzo[a]pyrene, by brown, red, green and chara algae have been studied (Kirso and Irha, 1998).

1.3.4. Limitations of biological treatment

There are certain limitations of using microbes for treating pollutants such as those high costs of production of microbial culture, limited mobility and survival of cells in the soil, alternative carbon source, completeness of the indigenous populations and metabolic inhibition (Husain and Jan, 2000; Duran and Esposito, 2000). Numerous organisms have been used for the complete degradation of aromatic compounds but much success has not been achieved yet (Robinson et al., 2001b; Keharia and Madamwar, 2003). Since bacterial, fungal and algal degradation of the aromatic compounds is attributed to secondary metabolic pathways, appropriate growth conditions have to be accomplished by additional loads of chemicals. Moreover, the expression of the enzymes involved in phenol, aromatic amines and dye degradation is not constant with time but dependent on the growth phase of the organisms and is influenced by inhibitors that may be present in the effluent (Wesenberg et al., 2003).

1.4. ENZYMATIC TREATMENT

In an attempt to overcome some of the problems associated with traditional chemical and biological waste treatment systems, recent research has focused on the environmental applications of enzymes that have been isolated from their parent organisms. Enzymatic systems fall between the two traditional categories of chemical and biological processes, since they involve chemical reactions based on the action of biological catalysts. Due to their high specificity to individual species or classes of compounds, enzymatic processes can be developed to specifically target selected compounds that are detrimental to the environment. Compounds that are candidates for this type of treatment are usually those that cannot be treated effectively or reliably using traditional techniques. Alternatively, enzymatic treatment can be used as a pretreatment step to remove one or more compounds that can interfere with subsequent downstream treatment processes. For example, if inhibitory or toxic compounds can be removed selectively, the bulk of the organic material could be treated biologically, thereby minimizing the cost of treatment. Due to the susceptibility of enzymes to inactivation by

the presence of other chemicals, it is likely that enzymatic treatment will be most effective in those streams that have the highest concentration of the target contaminant and the lowest concentration of other contaminants that may tend to interfere with enzymatic treatment (Ghioureliotis, 1997).

Before the full potential of enzymes may be realized, a number of significant issues remain to be addressed. Those include: development of low-cost sources of enzymes in quantities that are required at the industrial scale; demonstration of the feasibility of utilizing the enzymes efficiently under the conditions encountered during wastewater treatment; characterization of reaction products and assessment of their impact on downstream processes or on the environment into which they are released; and identification of methods for the disposal of solid residues, among others. Current research is focusing on addressing these issues, particularly for the development of enzymatic treatment systems that target the removal of aromatic compounds from industrial wastewaters.

The enzymatic tranformation of polluting compounds to less toxic or even innocuous products is an alternative to their complete removal. In this regard, a number of different redox enzymes are able to tranform a wide variety of toxic pollutants, such as phenols, azo dyes, PAHs, PCBs, heavy metals, etc. Peroxidases are the enzymes those have proved their potential in targeting different types of aromatic pollutants (Husain, 2006; Husain and Husain, 2007).

1.5. PEROXIDASES

Peroxidases catalyze oxidative transformations of organic reactants with peroxide as the oxidant. Usually the oxidant is H_2O_2, but alkylhydroperoxides, including *t*-butyl hydroperoxide, are also accepted by peroxidases. Based on their specific active center, peroxidases can be classified into three groups: heme peroxidases, vanadium peroxidases and non-metal peroxidases. Most well known are the heme peroxidases, which bear an iron protoporphyrin IX molecule at their active site (Adam et al., 1999). They have a molecular weight (M_r) ranging from 30-150 kDa and may be divided into mammalian and plant peroxidases. The family of plant peroxidases comprises yeast cytochrome c

peroxidase, plant ascorbate peroxidases, fungal peroxidases and other classical plant peroxidases, while the group of mammalian peroxidases includes myeloperoxidase, lacto peroxidase, thyroid peroxidase and prostaglandin H synthetase (Welinder, 1992).

WRF can oxidize various recalcitrant xenobiotics released to the environment by the human activity through the production of lignin modifying enzymes (LMEs). These extracellular LMEs have very low substrate specificity so they are able to mineralize a wide range of highly recalcitrant organopollutants that are structurally similar to lignin (Pointing, 2001; Cajthaml et al., 2002; Mansur et al., 2003; Veignie et al., 2004). The fact that these fungal enzymes work extracellularly allows them to access many of the non-polar and non-soluble toxic compounds (Reddy and Mathew, 2001; Levin et al., 2003). The three main LMEs are lignin peroxidase, Mn-dependent peroxidase, and laccase. All three of these enzyme groups are stimulated by nutrient limitation (Aust et al., 2003; Mansur et al., 2003).

Manganese peroxidases (MnP) are haemo-glycoproteins whose synthesis by fungi is induced by the presence of manganese^{2+} (Mn^{2+}) (Harayama, 1997). In nature, Mn^{2+} required for the induction and activity of MnP is supplied in the lignocellulose, which contains high levels of Mn^{2+} (Hofrichter et al., 1999). MnP catalyzes H_2O_2 dependent oxidation of Mn^{2+} to Mn^{3+} using phenolic compounds as a substrate and H_2O_2 as the terminal electron acceptor (Paszczynski and Crawford, 1995). MnP is thought to play the crucial role in the primary attack on lignin because it generates the strong oxidant Mn^{3+}; this oxidant acts as a diffusible redox mediator, which attacks certain aromatic moieties of the lignin polymer.

Lignin peroxidase (LiP) is a glycosylated heme protein that catalyzes H_2O_2 dependent oxidation of lignin-related aromatic compounds. They have a higher redox potential than most peroxidases and so are able to oxidize a wide range of chemicals, including some non-phenolic aromatic compounds (Reddy and Mathew, 2001).

The ligninolytic enzymes catalyze the degradation of pollutants by using a non-specific free radical mechanism (Pointing, 2001; Law et al., 2003). When an electron is added or removed from the ground state of a chemical it becomes highly reactive, allowing it to give or take electrons from other chemicals. This provides the basis for the non-specificity of the enzymes to degrade xenobiotics, chemicals that have never been

encountered in nature (Pointing, 2001; Reddy and Mathew, 2001). The main reactions that are catalyzed by the ligninolytic enzymes include depolymerization, demethoxylation, decarboxylation, hydroxylation and aromatic ring opening. Many of these reactions result in oxygen activation, creating radicals that perpetuate oxidation of the organopollutants (Reddy and Mathew, 2001). Once the peroxidases have opened the aromatic ring structures by way of introducing oxygen, other more common species of fungi and bacteria can mineralize the products intracellularly into products such as CO_2 and other benign compounds.

1.6. PEROXIDASE-MEDIATED TREATMENT OF ORGANOPOLLUTANTS

Table 1 summarizes the applications of peroxidases in the treatment and remediation of various aromatic compounds.

1.6.1. Polycyclic aromatic hydrocarbons

Several studies have shown that diverse WRF are capable of PAH mineralization, and rates of mineralization correlate with the production of LMEs (Sack et al., 1997). Experiments with purified cell-free enzyme extracts have confirmed the role of LMEs in PAH attack. The catalytic action of LiP and MnP generates more polar and water-soluble metabolites, such as quinones, which are more susceptible to further degradation by indigenous bacteria present in soils and sediments (Meulenberg et al., 1997).

The purified MnP (in a MnP-lipid peroxidation system) from *Phanerochaete chrysosporium* has been shown to oxidize twelve 3-6 ring PAH (Bogan and Lamar, 1995; Bogan et al., 1996a; 1996b; 1996c). Further evidence of lipid-peroxidation-coupled MnP-mediated PAH oxidation was observed for *Phanerochaete laevis*, which produced predominantly polar products, with no significant quinone accumulation (Bogan and Lamar, 1996). By contrast, MnP of *Nematoloma frowardii* (in a lipid-peroxidation-coupled reaction) oxidized anthracene and pyrene to produce quinone products (Guenther et al., 1998). These were not further metabolized, but some transformation of hydroxylated products occurred. The impact of Mn^{+2} supplementation and nutrient

limitation has been routinely found in MnP mediated PAH degradation. The effect of nutrient limitation on enzyme production was demonstrated by the increased production of MnP in nitrogen-limited media and its regulation by the amount of Mn^{+2} in the media. *Phanerochaete laevis* was found to have the capability to extensively transform anthracene, phenanthrene, benz[a]anthracene and benzo[a]pyrene (Bogan and Lamar, 1996). MnP was the predominant lignolytic activity detected in the supernatants of thirteen Deuteromycete lignolytic fungal strains investigated for their capacity to degrade PAHs and produce lignolytic enzymes during growth. This indicated that a wide range of fungi utilize this enzyme system for PAH degradation (Clemente et al., 2001).

Cajthaml et al. (2002) studied the ability of purified enzymes to degrade several PAHs. Levin et al. (2003) tested the biodegradation of two PAHs, nitrobenzene and anthracene, by a WRF, *Trametes versicolor*. They found high activities of MnP and laccase in the fungal cultures, suggesting that the enzymes are responsible for PAH degradation. MnP are thought to predominantly achieve the degradation of PAHs with lower ionization potentials and higher M_r. This has been demonstrated by the conversion of compounds such as benzo[a]pyrene, benzo [g,h,*i*]perylene and indenol[1,2,3-c,d]pyrene to larger extents than the higher ionization potential lower molecular mass PAHs such as phenanthrene and fluoranthene (Steffen et al., 2003). The action of MnP on lower ionization potential, high M_r PAHs was also demonstrated by the concurrent removal of 16 different PAHs in a cell free reaction mixture with crude enzyme or purified MnP. The ability to degrade these more recalcitrant higher M_r PAHs has led to the intensive investigation of MnP producing strains as potential inoculants for the bioremediation of high M_r PAHs (Steffen et al., 2003). The operation of MnP on PAHs does not necessarily occur independently. It is thought that in some fungi (*Deuteromycetes* and *Zygomycetes*), partial oxidation occurs through the MnP system prior to the involvement of intracellular reactions including cytochrome P-450 that interlock and enable efficient benzo[a]pyrene degradation (Steffen et al., 2003). The presence of organic acids has also been found to contribute to the degradation of PAHs in the basidiomycetous fungus *Nematoloma frowardii* (Hofrichter et al., 1999).

The development of an efficient degradation system for polyaromatics based on the use of peroxidases in vitro requires their increased bioavailability by using

cosolvents. Eibes et al. (2005) in a recent study have shown complete degradation of anthracene by MnP of *Phanerochaete chrysosporium* and *Bjerkandera* sp. BOS55 in organic solvent mixtures. Due to the maximal solubilization of anthracene and the minimum loss of MnP activity, acetone was selected as the optimal co-solvent, allowing to enhance 140-fold the anthracene solubility for an acetone concentration of 36% (v/v). In vitro degradation of anthracene by MnP was investigated for different concentrations of the main cofactors and substrates that affect the catalytic cycle of MnP (Mn^{2+}, H_2O_2 and organic acids) as well as for other environmental parameters; temperature, air/oxygen atmosphere and light source. MnP was purified using anion exchange and size exclusion chromatography from the WRF *Irpex lacteus*. Degradation of four representatives of PAHs (phenanthrene, anthracene, fluoranthene, and pyrene) was tested and the enzyme showed the ability to degrade them in vitro. Major degradation products of anthracene were identified. The results confirm the role of MnP in PAH degradation by *Irpex lacteus*, including cleavage of the aromatic ring (Baborova et al., 2006).

Extracellular preparations of LiP from *Phanerochaete chrysosporium* were among the first to be shown as capable of PAH oxidation (Haemmerli et al., 1986; Hammel et al., 1986; Bumpus, 1989). The WRF *Bjerkandera* sp. BOS55 produced more lignolytic peroxidases when grown in nitrogen-sufficient media. The absence of Mn was shown to repress MnP and stimulate LiP. Because the occurrence of LiP was associated with the highest anthracene degradation rates, it was considered a more efficient peroxidase towards anthracene than MnP (Kotterman et al., 1996). Purified LiP of *Nematoloma frowardii* has been shown to oxidize anthracene and pyrene in the presence of the mediator veratryl alcohol (VA) (Guenther et al., 1998). LiP-mediated PAH metabolism is thought to occur via one-electron oxidation to yield quinone products. The potential for the oxidation of substrates via LiP catalysis tends to correlate with their ionization potential. LiP has been shown to catalyze reactions (presumed to be oxidative ones) with PAH having ionization potentials of at or below 7.55 eV (Bogan et al., 1996a). Experiments using pyrene as a substrate in medium containing $H_2{}^{18}O$ showed that pyrene-1,6-dione and pyrene-1,8-dione are the major oxidation products, 84% of total and that the quinone oxygens come from water. The quinones formed were not substrates for LiP, hence the production of mutagens such as pyrene-1,6-dione and pyrene-1,8-dione

as well as some other quinones, that are not subject to further degradation, is of particular concern when exploiting this enzyme pathway (Hammel et al., 1986).

An 81 kDa protein from *Mycobacterium* sp. Strain PYR-1 was expressed in response to exposure of the strain to the PAH pyrene and recovered by two-dimensional gel electrophoresis. The N-terminal sequence of the protein indicated that it was similar to catalase-peroxidase (Wang et al., 2000). PAH oxidation using whole cells and purified manganese-lignin peroxidase (Mn LiP) from *Bjerkandera adusta* was studied. Oxidation of PAHs was examined by a purified Mn LiP hybrid isoenzyme in the presence and absence of manganese ions (Wang et al., 2003).

1.6.2. Polychlorinated biphenyls

Polychlorinated biphenyls are produced by chlorination of biphenyl, and many different congeners are produced which vary in their degree of substitution. The dehalogenation of PCBs by a commercial preparation of horseradish peroxidase (HRP) and purified laccase of *Trametes versicolor* has been demonstrated (Dec and Bollag, 1995), and this is probably due to LME-mediated free radical production. Furthermore, rates of peroxidase and laccase-mediated (*Trametes versicolor*) PCB attack were stimulated in the presence of 2,6-dimethoxyphenol co-substrate (Roper et al., 1995). Enzymatic transformation of different PCB congeners was studied using two oxidative enzymes, HRP and cytochrome c. The optimum-pH and H_2O_2 concentration for the maximum catalytic efficiency of the enzymes were pH 4.0 to 6.0 and 0.5 to 1.0 mM, respectively. Addition of polyethylene glycol (PEG) at low concentrations (50-100 ppm) to the reaction mixture had a significant protective effect on the activity of both enzymes and enhanced the transformation rate. Di-, tetra- and hexa-chlorinated biphenyls were better substrates as compared to tri- and penta-chlorinated biphenyls for HRP. Under optimum conditions the transformation efficiency of HRP was 60-80% depending on the congener (Singh et al., 2000).

1.6.3. Organochlorines

Organochlorine insecticides such as 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), lindane, and the aldrins (aldrin, dieldrin, endosulfan) have been manufactured and applied in vast quantities since 1940s. Organochlorine herbicides such as 2,4-dichlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid, and 2-methyl-4,6-dichlorophenoxyacetic acid have also been widely used, with the concomitant generation of dioxins. The potential of peroxidase for soil and water detoxification constitute a possible basis for the development of bioremediation technologies. Biodegradation of DDT can result in the formation of toxic and persistent metabolites. The mineralization of the dioxin 2,7-dichlorodibenzo-*p*-dioxin by *Phanerochaete chrysosporium* has been demonstrated. In this study purified LiP and MnP were capable of mineralization in a multi-step pathway involving sequential oxidation, reduction and methylation reactions to remove the two Cl atoms and carry out ring cleavage (Valli et al., 1992).

The herbicide atrazine was biotransformed to the less toxic compounds deethylatrazine and hydroxyatrazine, N-dealkylated and hydroxylated metabolites, respectively, by *Phanerochaete chrysosporium*, in a liquid culture. The activity of LiP and MnP coincided with the degradation of atrazine (Mougin et al., 1994). The herbicide bentazon (3-isopropyl-1 H-2,1,3-benzothiadiazine-4(3H)-one-2,2-dioxide), a relatively inert chemical, and some of its metabolites were incubated with HRP in the presence or absence of humic monomers to evaluate the incorporation of herbicide and its metabolites into humic material by oxidative enzymes. At pH 3.0, in the presence of peroxidase and guaiacol, the concentrations of bentazon and its metabolites 2-amino-N-isopropyl-benzamide, des-isopropyl-bentazon and 8-chloro-bentazon decreased by 9, 70, 30 and 5%, respectively. In the presence of HRP, bentazon was transformed 90% by catechol, 94% by vanillic acid, 65% by protocatechuic acid, 49% by syringaldehyde and 27% by caffeic acid (Kim et al., 1998). A soil sample containing a triazine (hexahydro-1,3,5-trinitro-1,3,5-triazine) was degraded by a bacterial strain. The resulting ring cleavage product (4-nitro-2,4-diazabutanal) was used as a substrate by *Phanerochaete chrysosporium*, leading to its removal with the release of nitrous oxide. Further

experiments showed that MnP was responsible for this degradation (Fournier et al., 2004).

1.6.4. 2,4,6-trinitrotoluene

A two-step process for the removal of dinitrotoluene from water is presented: zero-valent iron reduction is coupled with peroxidase-catalyzed polymerization of the resulting diaminotoluenes (DAT). The effect of pH was examined in the reduction step: at pH 6 the reaction occurred much more rapidly than at pH 8. In the second step, optimal pH and substrate ratio, minimal enzyme concentration and effect of PEG as an additive for greater than 95% conversion of DAT, over a 3 h reaction period were determined using HPLC. Two enzymes were investigated and compared: *Arthromyces ramosus* peroxidase (ARP) and soybean peroxidase (SBP). The optimal pH was 5.4 and 5.2 for ARP and SBP, respectively, but SBP was more resistant to mild acid whereas ARP was more stable in neutral solutions. SBP was found to have a greater H_2O_2 demand (optimal peroxide/DAT molar ratio for SBP: 2.0 and 3.0 for 2,4-diaminotoluene (2,4-DAT) and 2,6-diaminotoluene (2,6-DAT), respectively; for ARP: 1.5 and 2.75 for 2,4-DAT and 2,6-DAT, respectively) but required significantly less enzyme (0.01 and 0.1 U mL^{-1} for 2,4-DAT and 2,6-DAT, respectively) to convert the DAT than ARP (0.4 and 1.5 U mL^{-1} for 2,4-DAT and 2,6-DAT, respectively). PEG was shown to have no effect upon the degree of substrate conversion for either enzyme (Patapas et al., 2007).

Ligninolytic enzymes were also shown to participate actively during the oxidative degradation of TNT (Van Aken et al., 1997; 1999; Kim and Song, 2000). In similar studies Hawari et al. (1999) observed TNT mineralization when LiP and MnP were detectable in the culture medium of *Phanerochaete chrysosporium*. Van Aken et al. (2000) demonstrated that TNT was first reduced, most probably by nitroreductases in the fungal mycelium to aminodinitrotoluenes and diaminonitrotoluenes. In the second phase, the reduced TNT metabolites were oxidatively transformed and mineralized. A purified LiP, H 8 from *Phanerochaete chrysosporium* was capable to completely transform 50 mg L^{-1} of 2,4-diamino-6-nitrotoluene and 2-amino-4,6-dinitrotoluene in 1 and 48 h, to nitrosodinitrotoluenes. No significant mineralization to $^{14}CO_2$ was observed. Lee et al.

(2001) developed an *in situ* H_2O_2 generating system, which was used by LiP for the degradation of wastewater containing TNT. The efficiency of removal of TNT was greater than that of biochemical methods under optimal conditions. Denitrification was proportional to the amount of TNT degraded. The electroenzymatic method had a lower power requirement than electrochemical oxidation at -0.2 and -0.4 V.

Scheibner et al. (1997) showed that MnPs from *Nematoloma frowardii* and from the litter decaying *Stropharia rugoannulata* were able to transform TNT reduction products. They observed mineralization of ring labeled ^{14}C-2-amino-2,6-dinitrotoluene directly to CO_2. In the cell free system, a release of 52% $^{14}CO_2$ was demonstrated in the presence of reduced glutathione as a secondary thiol mediator. Hofrichter et al. (1999) demonstrated enzymatic combustion of aromatic and aliphatic compounds by MnP from *Nematoloma frowardii*. Mineralization was dependant on the ratio of MnP activity to concentration of reduced glutathione, a finding demonstrated by using ^{14}C-2-aminodinitrotoluene as an example. Degradation of TNT by MnP from *Phlebia radiata* in the presence of reduced glutathione was further investigated by few workers (Van Aken et al., 1999). Van Aken and Agathos (2002) developed an abiotic system (Mn^{+3}, oxalate, and oxygen) for degradation of nitroaromatic compounds by MnP.

1.6.5. Bleach-plant effluent

The production of high quality paper requires a chlorine-mediated bleaching process to remove color associated with the 5-10% residual lignin in pulp. As a result, large aqueous volumes of toxic, low molecular mass, halogenated lignin degradation products are released into the environment from bleach-plants. These include chloro-lignins, chloro-phenols, chloro-guaiacols, chloro-catechols, and chloro-aliphatics (Neilson et al., 1991).

Oxidative demethylation and dechlorination of bleach-plant effluent, with associated decolorization, has been demonstrated for *Phanerochaete chrysosporium* (Michel et al., 1991; Jaspers et al., 1994). Success of the decolorization of paper mill bleach plant effluent has also been reported using unidentified marine fungi, which produced enzymes; MnP, LiP and laccase (Raghukumar et al., 1996). Enzyme pulping

using MnP and laccase of *Polyporus* sp. and pectinases from *Rhizopus* sp. 26R significantly reduced the amount of NaOH in a following alkaline pulping process (Poonpairoj et al., 2001). A crude MnP preparation from the marine basidiomycete *Phlebia* sp. MG 60, effectively bleached a hard wood kraft pulp (UKP). When a model effluent 'white-water' was used to bleach UKP, the pulp treated with MnP from *Phlebia* sp. was brighter than that achieved using MnP from *Phanerochaete chrysosporium* (Kondo et al., 2002). Thus, MnP from *Phlebia* sp. is potentially useful in biobleaching when white-water is recycled. Major lignin-degrading enzymes from basidiomycetes include MnP, laccase and to a lower extent LiP (Hatakka et al., 2002). MnP in the presence of Mn^{+2} chelated with an organic acid, Tween 80, and a H_2O_2 generating system depolymerized milled pine wood (Hatakka et al., 2003). A key element in the delignification system is Mn^{+3}, a strong oxidizing agent that is generated by MnP (Feijoo et al., 2002).

1.6.6. Phenolic compounds

In recent years, a great deal of research has been directed towards developing processes in which peroxidases are used to remove phenolic contaminants from toxic wastewater (Klibanov et al., 1983; Tatsumi et al., 1996; Duran and Esposito, 2000). Phenols are oxidized by peroxidases to generate phenoxy radicals, which couple to form oligomeric and polymeric products (Ward et al., 2001). The polymerization reaction can be written as follows:

$$H_2O_2 + 2AH_2 \xrightarrow{\text{Peroxidase}} 2H_2O + \text{polymerized products (HAAH)}$$

HAAH can serve as a hydrogen donor, leading to higher degree of polymerization. These polymeric products have limited water solubility and tend to precipitate quite readily (Tatsumi et al., 1996; Duran and Esposito, 2000).

Extensive investigations have been conducted on reaction kinetics, model development, and optimization of the polymerization reaction using HRP (Nicell et al., 1993; Wu et al., 1998; Buchanan and Nicell, 1998). HRP has received particular attention because of its catalytic ability under a wide range of conditions such as temperature, pH

and contaminant concentrations and because its catalytic function is well understood (Karam and Nicell, 1997).

Laurenti et al. (2003) have investigated the reaction mechanism of the oxidation of 2,6-DCP by HRP and H_2O_2 and the reaction products were characterized by UV-visible spectrophotometry and mass spectrometry. Aqueous solutions of five different phenols were treated with H_2O_2 and HRP resulting in more than 95% removal of phenols within 3 h (Wagner and Nicell, 2002). Foul condensate originating from the kraft recovery process, containing phenolic compounds was treated with HRP. The treatment selectively targeted phenolic compounds resulting in a reduction of the total phenol concentration below 1 mg L^{-1} (Wagner and Nicell, 2003). Won et al. (2004) have investigated the HRP catalyzed polymerization of cardanol in aqueous organic solvent in the presence of a redox mediator. FT-IR and GPC analysis of the product revealed that the structure and properties of polycardanol formed by HRP with a mediator were similar to the product formed by SBP. Sonolysis, enzyme treatment, and a combination of the two processes were tested for the degradation of phenol in aqueous medium. With sonolysis alone, 423 kHz ultrasound eliminated phenol molecules through hydroxyl radical reactions. In the presence of H_2O_2, the enzyme HRP catalyzed the oxidation of phenol to insoluble polymers, which could be separated easily from the solution. In the combined system; HRP, H_2O_2, US-423 kHz, the degradation rate was accelerated, and the color of the solution changed to dark brown without any precipitate formation (Entezari and Petrier, 2004).

The majority of reports on detoxification of wastewater contaminated with phenols, cresols, and chlorophenols, have used HRP. However, recently peroxidases from other sources such as soybean (Caza et al., 1999; Kinsley and Nicell, 2000; Kennedy et al., 2002), turnip (Duarte-Vazquez et al., 2002) and bitter gourd (Akhtar and Husain, 2006) have been suggested as alternatives to horseradish. The *Coprinus cinereus* peroxidase (CIP) was used for the removal of ten different phenols from water. The treatment of aqueous solution of phenol with the fungal peroxidase resulted in the polymerization and precipitation of the phenols (Kauffmann et al., 1999). Wright and Nicell (1999) investigated the treatment of several aqueous phenolic compounds by SBP. Duarte-Vazquez et al. (2002) investigated the efficiency of TP in removing different

phenolic compounds. Enzymatic treatment of a strong oil refinery wastewater was investigated using crude CIP from *Coprinus cinereus* UAMH 4103 and H_2O_2. Phenolic compounds in the refinery wastewater were enzymatically converted to coloured polymeric products, which were subsequently removed by coagulation with alum (Ikehata et al., 2003).

The effects of extracts from artichoke (*Cynara scolymus* L.) flower bracts on model wastewaters containing a range of phenolic contaminants have been studied (Lopez-Molina et al., 2003). The extracts contained various isoenzymes of both peroxidase and polyphenol oxidase. HPLC measurements showed that the monophenol and 4-chlorophenol were most effectively oxidized in the presence of both extract and H_2O_2, suggesting that this substrate was mainly being acted upon by peroxidase. Recently Bodalo et al. (2006) described a comparison between HRP and SBP; the two most widely used commercial peroxidases for the removal of phenols from wastewater.

The major obstacle in the commercial applications of peroxidases for environmental purposes is their limited operational stability, which means that a continuous supply of large amounts of fresh enzyme is required. Heme peroxidases are susceptible to H_2O_2-dependent, as well as product dependent inactivation (Hiner et al., 1995; Van de Velde et al., 2001). In order to minimize such inactivation, some researchers have suggested adding compounds such as PEG to decrease the adsorption of polymer onto the enzyme's active site. The addition of PEG has been shown to greatly reduce the amount of enzyme required, thus increasing the competitiveness of the process (Wu et al., 1997). It has been demonstrated that PEG combines with the polymerization products formed during the reaction because it has a higher partition affinity with the polymer products than peroxidases (Wu et al., 1998). Cooper and Nicell (1996) showed that PEG addition increased removal efficiency of a foundry wastewater using HRP. Addition of PEG-3350 or PEG-8000 to SBP catalyzed reaction, increased the removal efficiency of 2,4-dichlorophenol by a factor of 10 or 50, respectively (Kennedy et al., 2002).

The addition of PEG could significantly enhance the phenol removal efficiency, and reduce the amount of immobilized enzyme required to achieve a high removal efficiency of over 90%. When the mass ratio of PEG/phenol and the molar ratio of

H_2O_2/phenol were 0.4 and 1.5, respectively, the oxidation of phenol could be completed within short time after the initiation of reaction in the absence of buffer using HRP immobilized on Al-PILC. Studies were carried out in the absence and presence of different concentrations of a stabilizing additive agent, PEG, in an attempt to optimize the phenol elimination procedure by HRP and SBP (Bodalo et al., 2006). The final choice of peroxidase will depend on the characteristics of the effluent, operational requirements and on economic considerations. Their findings show that HRP acts faster than SBP but is more susceptible to inactivation, although PEG better protects it. In consequence HRP will be the most suitable choice but the addition of a sufficient amount of PEG is critical.

1.6.7. Aromatic amines

LiP from the WRF *Phanerochaete chrysosporium* was chemically modified by reductive alkylation with benzyl, naphthyl and anthracyl moieties, thereby increasing its superficial hydrophobicity. The three chemical modifications altered the kinetic behaviour of the enzyme in 10% acetonitrile with four different substrates: carbazole, pinacyanol, pyrene and veratryl alcohol. Benzyl modification of lignin peroxidase increased the catalytic efficiency ($k_{cat}/K_{m,app}$) 2.7 times for carbazole oxidation. Thirteen N-containing compounds, including pyrroles, pyridines, and aromatic amines, were tested to determine whether they could be oxidized by lignin peroxidase in 10% acetonitrile. All pyrrole analogues and amines tested were oxidized, but none of the pyridine analogous reacted. Some products were isolated and analyzed by high-resolution mass spectrometry. Most were dimers or polymers and, in some cases, these contained oxygen atoms (Vazquez-Duhalt et al., 1995).

1.6.8. Synthetic dyes

Some earlier workers have shown that oxidative destruction of colored compounds was significantly stimulated by oxidative enzymes (Bhunia et al., 2001; Shaffiqu et al., 2002; Mohan et al., 2005), and may be of practical interest for the decolorization of synthetic dyes. Oxidative enzymes those involved in the ligninolytic

systems of WRF are the most important class of enzymes suited for enzymatic dye degradation (Mester and Tien, 2000; Duran and Esposito, 2000). These enzymes can be produced at a large scale and may be applied in crude form (Linko, 1988; Moreira et al., 1998). Besides laccases (Abadulla et al., 2000), peroxidases seem to be the most promising enzymes.

Bhunia et al. (2001) showed that HRP was quite effective in degrading and precipitating industrially important azo dyes. They evaluated the specificity of HRP toward different dyes, such as Remazol Blue and Cibacron Red. For Remazol Blue, the enzyme activity was found to be far better at pH 2.5 than at neutral pH. Shaffiqu et al. (2002) have used peroxidases from plants *Ipomea palmata* and *Saccharum spontaneum* as an alternative to the commercial available horseradish and soybean peroxidase for the decolorization of textile dyes, mainly azo dyes. Eight textiles dyes currently used by the textile industry and seven other dyes (25-200 mg L^{-1}) were selected for decolorization, using these plant enzymes. Purified peroxidase of *S. spontaneum* leaf could completely degrade Supranol Green and Procion Green HE-4BD (100%) dyes within 1 h, whereas Direct Blue, Procion Brilliant Blue H-7G and Chrysoidine were degraded more than 70% in 1 h. Peroxidase of Ipomea (*I. palmata* leaf) degraded 50 mg L^{-1} of the dyes; Methyl Orange (26%), Crystal Violet (36%), and Supranol Green (68%) in 2-4 h and Brilliant Green (54%), Direct Blue (15%), and Chrysoidine (44%) at the 25 mg L^{-1} level in 1 to 2 h of treatment. Catalase-peroxidase from thermoalkaliphilic *Bacillus* sp. exhibited very high potential in the treatment of textile bleaching effluents (Gudelj et al., 2002).

Akhtar et al. (2005a) have investigated the potential of a peroxidase from *Momordica charantia* in decolorizing industrially important dyes. Dye solutions (50-200 mg L^{-1}) were used for the treatment. *Momordica charantia* peroxidase was able to decolorize most of the textile dyes by forming an insoluble precipitate. The greater fraction of the color was removed when the dyes were treated with increasing concentrations of enzyme but four out of eight reactive dyes were recalcitrant to decolorization by BGP. Decolorization of non-textile dyes resulted in their degradation and removal from the solution without any precipitate formation. Mohan et al. (2005) showed the effectiveness of HRP catalyzed enzymatic reaction in the treatment of an acid azo (Acid Black 10 BX) dye. However, the performance of HRP catalyzed reaction was

found to be dependent upon the reaction time, dye and HRP concentrations, H_2O_2 dose and aqueous phase pH.

More recently some investigators have reported that microperoxidase-11 (MP-11), a heme-containing undecapeptide, derived from horse heart cytochrome C was utilized as a peroxidative catalyst (Wariishi et al., 2002). The decolorization of water-insoluble synthetic dyes by MP-11 in 90% methanol was attempted. MP-11 exhibited effective decolorization activity against azo or anthraquinone types dyes. The degradation pathway for Solvent Orange 7 was investigated in detail, showing that MP-11 catalyzed the oxidative cleavage of the azo linkage to generate 1,2-naphthoquinone and 2,4-dimethylphenol as key intermediates. Shin et al. (1997) showed that the edible macroscopic fungi *Pleurotus ostreatus* produced an extracellular peroxidase that can decolorize RBBR. In a later report, Shin and Kim (1998) studied the efficiency of a *Pleurotus ostreatus* peroxidase on the decolorization of dyes belonging to four structurally different groups: triarylmethane, heterocyclic, azo, and polymeric. All dyes tested were partially decolorized, where the best decolorization, 98% was achieved for Bromophenol Blue, while heterocyclic dyes, Methylene Blue and Toluidine Blue O, were the least decolorized (10%).

MnP from *Phanerochaete chrysosporium* was also able to decolorize several azo dyes in vitro, and with both enzymes the decolorization rate was dependent on the chemical structure of the dye (Pasti-Grigsky et al., 1992). The MnP of *Bjerkandera adusta* and *Pleurotus eryngii* have also been shown to catalyze dye decolorization (Heinfling et al., 1998a). The enzymes from both fungi were unusual in that they did so in Mn^{+2} independent reaction (i.e. the Mn^{+3} lactate complex was not the oxidative agent created by the enzyme). Moreira et al. (2001) have evaluated an enzymatic action of the ligninolytic enzyme MnP, through a suitable addition of H_2O_2, as a feasible system for the *in vitro* degradation of complex structures. The highly recalcitrant polymeric dye (Poly R-478) was selected as a model compound. An amperometric technique was used to determine the H_2O_2 requirement in the decolorization by non-purified MnP. In this study two modes of H_2O_2 supply, fed-batch (every h) or semicontinuous (every 5 min), were applied. The enzymatic treatment catalyzed not only the destruction of the

chromophoric groups but also a noticeable breakdown of the chemical structure of the dye.

The production of manganese-dependent peroxidase by *Phanerochaete chrysosporium* in a new solid-state bioreactor, the immersion bioreactor, operating with lignocellulosic waste, such as wood shavings, was investigated. The *in vitro* decolorization of several synthetic dyes by the extracellular liquid produced in the above-mentioned bioreactor, containing mainly MnP, was carried out and its degrading ability was examined. The highest decolorization was reported for Indigo Carmine 98% followed by Bromophenol Blue 56% and Methyl Orange 36%, whereas Gentian Violet was marginally decolorized to 6% (Rodriguez-Couto et al., 2002). An immobilized *Phanerochaete chrysosporium* bioreactor operated with pulsation of the gas-phase was proposed and operated at variable conditions for the continuous biological degradation of a recalcitrant dye Poly R-478. A high percent of decolorization, between 65% and 80% under optimal conditions was achieved. Moreover, the system proved to have high stability with long operational periods of at least 90 d. During the operation MnP was the sole ligninolytic enzyme responsible for decolorization of dye (Mielgo et al., 2002). MnP was reported as the main enzyme of *Clitocybula dusenii* involved in the breakdown of the dyes in the real dye-containing effluent. The activity of MnP increased commensurately with the proportion of the raw dye wastewater in the medium. Maximal MnP activity (180 U L^{-1}) was found after 20 d using 33% raw effluent (Wesenberg et al., 2002).

MnP was produced by shallow stationary cultures of *Phanerochaete chrysosporium* growing on N-limited medium. Decolorization of sulfonaphthalein (SP) dyes by MnP was investigated. The MnP activity profile and decolorization of SP dyes was correlated and almost all dyes were decolorized at pH 4.0. The influence of various inhibitors on Bromocresol Purple decolorization suggested an oxidative nature of the MnP-catalyzed decolorization of SP dyes (Christian et al., 2003). Moldes et al. (2003) studied the production of MnP and LiP by *Phanerochaete chrysosporium* in a fixed-bed tubular bioreactor, filled with cubes of nylon sponge, operating in semi-solid-state conditions. The *in vitro* decolorization of two structurally different dyes; Poly R-478 and Crystal Violet by the extracellular liquid obtained in the above mentioned bioreactor was monitored in order to determine its degrading capability. A methodology for a long

treatment of these dyes based on the continuous addition of MnP and H_2O_2 resulted in a decolorization of 70% and 30% for Crystal Violet and Poly R 478 after 2 h, respectively.

Mielgo et al. (2003) aimed to develop a degradation system based on the use of the ligninolytic enzyme MnP for the degradation of azo dyes. This work included the optimization of the process, with the objective of determining the influence of specific physicochemical factors, such as organic acids, H_2O_2, Mn^{2+} concentration, pH, temperature, enzyme activity and dye concentration. A nearly total decolorization was possible at very low reaction times, 10 min and at high dye concentration (up to 1500 mg L^{-1}). A specific oxidation capacity as high as 10 mg dye degraded per unit of MnP consumed was attained for a decolorization higher than 90%. Among all, the main factor affecting process efficiency was the strategy of H_2O_2 addition. The continuous addition at a controlled flow permitted the progressive participation of H_2O_2 in the catalytic cycle through a suitable regeneration of the oxidized form of the enzyme, which enhanced both extent and rate of decolorization. *Irpex lacteus*, a WRF, was effective in the decolorization of textile industrial wastewater without adding any chemicals. The degree of decolorization of the dye effluent by shaking and stationary cultures was 59 and 93%, respectively, on the 8th d. The higher level of manganese-dependent peroxidase and non-specific peroxidase (NsP) was detected in stationary cultures rather than in the shaken cultures. The absorbance of the dye effluent was significantly decreased by the stationary culture filtrate of 7 d in the absence of Mn (II) and veratryl alcohol (Shin, 2004).

Boer et al. (2004) demonstrated the ability of WRF, *Lentinula edodes*, to decolorize several synthetic dyes using solid-state cultures with corncob as substrate. Cultures, containing Amido Black, Congo Red, Trypan Blue, Methyl Green, RBBR, Methyl Violet, Ethyl Violet and Poly R 478 at 200 ppm, were completely decolorized after 18 d of incubation. Partial decolorization was observed in the cultures containing 200 ppm of Brilliant Cresyl Blue and Methylene Blue. High MnP activity but very low LiP and laccase activities were detected in the cultures. *In vitro*, the dye decolorization was remarkably decreased in the absence of Mn^{+2} and H_2O_2. These findings suggested that the MnP appeared to be the main enzyme, responsible for the decolorization of synthetic dyes. Some investigators have developed enzymatic membrane reactors (EMR) for the oxidation of azo dyes by MnP (Song et al., 2002; Lopez et al., 2002; 2004). The

configuration consisted of a stirred tank reactor coupled with an ultrafiltration membrane. The membrane allowed for most of the enzymatic activity to be recovered while both the parent dye and the degradation products could pass through. Different operational strategies (batch, fed-batch, and continuous) and parameters such as enzyme activity, H_2O_2, feeding rate, hydraulic retention time (in continuous operation), and dye loading rate were studied (Lopez et al., 2004). Under the best conditions, continuous operation with a dye decolorization higher than 85% and minimal enzymatic deactivation was feasible for 18 d, attaining an efficiency of 42.5 mg Orange II oxidized/MnP unit consumed.

Verma and Madamwar (2005) in a recent study reported that basidiomycete PV002, a white-rot strain from decomposed neem waste displayed high extracellular peroxidase activity and rapidly decolorized azo dyes. White-rot strain PV002 efficiently decolorized Ranocid Fast Blue (96%) and Acid Black 210 (70%) on 5 and 9 d, respectively under static conditions. The degradation of azo dyes under different conditions was strongly correlated with high MnP activity. Machado et al. (2005) used RBBR dye as substrate to evaluate ligninolytic activity in 125 basidiomycetous fungi isolated from tropical ecosystems. Extracellular extracts of 30 selected fungi grown on solid medium with sugar can bagasse showed RBBR decolorization and peroxidase activity. Eight fungi produced MnP activity with expressive RBBR decolorization capacity. Eichlerova et al. (2006) tested eight different *Pleurotus* species for their Orange G and RBBR decolorization capacity and their ligninolytic properties. Strain CCBAS 461 of a very little studied species *Pleurotus calyptratus* was chosen for a more detailed study. This strain produced a relatively high amount of MnP activity. Within 14 d the strain decolorized up to 91% of Orange G and 85% of RBBR in liquid culture and more than 50% of these dyes on agar plates. *Pleurotus calyptratus* is able to decolorize efficiently also other azo and phthalocyanine dyes, but only a limited decolorization capacity was found in the case of polyaromatic and triphenylmethane dyes. Litter-decomposing basidiomycete fungi (LDF) including environmental isolates from oak forest soil were compared with WRF for ligninolytic enzymes production and decolorization of synthetic dyes Poly B-411, Reactive Black 5, Reactive Orange 16 and RBBR. The highest activity of MnP was detected in the cultures of *Collybia dryophila*

with the peak activity over 30 U L^{-1}. The fastest degradation of Poly B-411 was performed by the strains with high levels of MnP and laccase while the decolorization of other dyes did not depend so strictly on enzyme activity (Baldrian and Snajdr, 2006).

The decolorization of six commercial dyes with 10 fungal strains was studied. Enzyme activity, decolorization trends and decolorization mechanisms were monitored. Under the experimental conditions, MnP and extracellular laccase, but not LiP, were detected. The decolorization mechanisms by *Funalia trogii* involved a complex interaction of enzyme activity and biosorption (Park et al., 2007). Purified MnP from *Ischnoderma resinosum* performed decolorization of all textile dyes tested; Reactive Black 5, Reactive Blue 19, Reactive Red 22 and Reactive Yellow 15. The highest decolorization rates were detected in acidic pH (Kokol et al., 2007). The decolorization of 12 different azo, diazo and anthraquinone dyes was carried out using a new isolated WRF, strain L-25. A decolorization efficiency of 84.9–99.6% was achieved by cultivation in 14 d using an initial dye concentration of 40 mg L^{-1}. The activity of MnP in the cultures was over 1.0 U mL^{-1} at the end of cultivation. MnP produced by strain L-25 was used for the enzymatic decolorization of the dyes thus confirming the capability of the enzyme for this purpose (Reza et al., 2007). In a recent study, MnP was isolated and purified from *Schizophyllum* sp. F17 on pinewood solid-state cultures. The azo dyes such as Congo Red, Orange G and Orange IV were efficiently decolorized using the purified MnP (Cheng et al., 2007).

The chemical steps involved in the degradation of azo dyes by LiP and MnP has been elucidated (Pasti-Grigsby et al., 1992; Goszczynski et al., 1994). The mechanism of azo dye oxidation by peroxidases such as LiP probably involves the oxidation of phenolic groups to produce a radical at the carbon bearing the azo linkage. Then water attacks this phenolic carbon to cleave the molecule producing phenyldiazine. The phenyldiazine can be oxidized by a one-electron reaction generating N_2 (Spadaro and Renganathan, 1994; Chivukula et al., 1995). Extracellular fluid from cultures of *Phanerochaete chryosporium* and purified LiP were able to degrade Crystal Violet and 6 other triphenylmethane dyes by sequential N-demethylation (Bumpus and Brock, 1988). LiP of *Phanerochaete chryosporium* has been shown to decolorize azo, triphenylmethane and heterocyclic dyes in the presence of VA and H_2O_2 (Cripps et al., 1990; Ollikka et al., 1993). Ollikka et al.

(1993) reported 75% decolorization of RBBR using crude LiP preparation. However, in this other agents such as VA or even enzyme may have contributed to the decolorization. Chivukula and Renganathan (1995) have shown oxidation of sulphonated dyes by using LiP.

Decolorization of eight synthetic dyes including azo, anthraquinone metal complex and indigo was examined in WRF cultures by peroxidase catalyzed oxidation (Young and Yu, 1997). Above 80% color was removed by LiP catalyzed oxidation, further dye decolorization rate increased linearly with LiP dosage. Three LiP isoenzymes, LiP 3.85, LiP 4.15, and LiP 4.65, were purified from the carbon-limited culture medium of *Phanerochaete chrysosporium.* The purified isoenzymes were used for decolorization of an azo dye, Crocein Orange G (COG). According to the kinetic data obtained, the oxidation of COG by LiP appeared to follow Michaelis-Menten kinetics. Kinetic parameters for each isoenzyme were determined (Ollikka et al., 1998).

Peralta-Zamora et al. (1999) have also described that LiP from *Phanerochaete chrysosporium* could decolorize about 30% RBBR in a 1.0 ml reaction volume but scaling up the reaction was not used for large scale applications because unrealistically large amount of enzyme would be required. LiP was reported as the main enzyme involved in dye decolorization by *Bjerkandera adusta* (Robinson et al., 2001a) and *Phanerochaete chrysosporium* (Kirby et al., 1995). Verma and Madamwar (2002) demonstrated the decolorization by partially purified LiP from *Phanerochaete chrysosporium* grown on neem hull waste. This enzyme decolorized 80% Procion Brilliant Blue HGR, 83% Ranocid Fast Blue, 70% Acid Red 119 and 61% Navidol Fast Black MSRL. The effects of different concentrations of VA, H_2O_2, enzyme and dye on the efficiency of decolorization have been investigated. In order to achieve an effective decolorization with *Funalia trogii*, fungal immobilization and repeated batch experiment were investigated (Park et al., 2006). The activities of three enzymes, namely LiP, MnP and laccase, were reported during decolorization of Acid Black 52 under the condition of immobilized and suspended fungal mycelia. Decolorization could be stably maintained with the repeated batch experiments during long period (at least for 300 h).

Yu et al. (2006) carried out production of ligninolytic enzymes at C/N ratios of 28/44 mM and 56/2.2 mM in nonimmersed liquid culture of *Phanerochaete*

chrysosporium. In vitro decolorization of one industrial azo dye, Reactive Brilliant Red K-2BP, by crude LiP and MnP obtained under carbon and nitrogen limitation respectively, was examined and compared for their degradation characteristics. Decolorization by both LiP and MnP were sensitive to pH, peaking around pH 3.0, and improved at higher enzyme activities. Decolorization by LiP can be enhanced to the greatest degree (89%) with higher addition of H_2O_2 and VA, whereas decolorization by MnP was optimized only with a suitable dose of H_2O_2 (0.1 mM) and decreased by the addition of Mn^{2+}. Decolorization declined at high dye concentrations; LiP was able to decolorize a dye concentration of 60 mg L^{-1} and below to no less than 85%, and MnP of 10 mg L^{-1} to a maximum of 71%. Combined decolorization by LiP and MnP was somewhat lower than that by LiP alone. It was suggested that the optimization of the H_2O_2 supply was mainly responsible for a high efficiency in continuous dye degradation by crude LiP and MnP. *Pseudomonas desmolyticum* NCIM 2112 was able to degrade a diazo dye Direct Blue 6 (100 mg L^{-1}) completely within 72 h of incubation with 88.95% reduction in COD in static anoxic condition. The decolorization and degradation of dye in the batch culture represents the role of LiP, MnP and laccase. The final products, 4-amino naphthalene and amino naphthalene sulfonic acid were characterized by GC–mass spectroscopy (Kalme et al., 2007).

Reports on decolorization through enzymatic processes by yeast or other filamentous fungi different from WRF are limited. A purified peroxidase produced by *Geotrichum candidum* Dec 1, was involved in decolorization of dyes (Kim and Shoda, 1999). This peroxidase was produced under aerobic conditions as a secondary metabolite in the stationary phase. Nine of the 21 dyes decolorized by the cells of *Geotrichum candidum* Dec 1, were also decolorized by the purified peroxidase, in particular anthraquinone dyes. Sugano et al. (2000) have demonstrated the efficient expression of the dye-decolorizing peroxidase, DyP, from *Geotrichum candidum* Dec 1 in *Aspergillus oryzae* M-2-3. Further these workers have constructed an expression system of a unique dye-decolorizing peroxidase, DyP in *Escherichia coli*. The molecular mass of the expressed DyP (eDyP) was 47 kDa, indicating no modification with saccharides. The characteristics of eDyP were almost the same as those of native DyP from a fungus *Thanatephorus cucumeris* Dec 1 and recombinant DyP with *Aspergillus oryzae* except

Table 1: Remdiation of various organopollutants by peroxidases

Enzyme	Source	Substrate	Ref.
MnP	*Phanerochaete chrysosporium*	3-6 ring PAHs	Bogan and Lamar, 1995; Bogan et al., 1996a; 1996b; 1996c
	Phanerochaete laevis	PAH	Bogan and Lamar, 1996
	Nematoloma frowardii	Anthracene, Pyrene	Guenther et al., 1998
		PAHs	Hofrichter et al., 1999
	Phanerochaete chrysosporium	Anthracene	Eibes et al., 2005
	Bjerkandera spBOS55		
	Irpex lacteus	phenanthrene, anthracene, fluoranthene, and pyrene	Baborova et al., 2006
MnP+lac	*Trametes versicolor*	Nitrobenzene and anthracene,	Levin et al., 2003
LiP	*Phanerochaete chrysosporium*	PAH	Haemmerli et al., 1986; Hammel et al., 1986; Bumpus, 1989
	Bjerkandera spBOS55	Anthracene	Kotterman et al., 1996
	Nematoloma frowardii	Anthracene, Pyrene	Guenther et al., 1998
Mn LiP	*Bjerkandera adusta*	PAHs	Wang et al., 2003
POX + laccase	Horseradish	PCBs	Dec and Bollag, 1995; Roper et al., 1995
POX + cytochrome c		Different PCB congeners	Singh et al., 2000
POX	Horseradish	Bentazon	Kim et al., 1998
MnP	*Phanerochaete chrysosporium*	4-nitro-2,4-diazabutanal	Fournier et al., 2004
LiP + MnP	*Phanerochaete chrysosporium*	Dioxin	Valli et al., 1992
		Atrazine	Mougin et al., 1994
MnP	*Phlebia* sp. MG60	Hard Wood Kraft pulp	Kondo et al., 2002
	Phanerochaete chrysosporium		
	Basidiomycetes	Milled pine Wood	Hatakka et al., 2003
MnP + LiP and Laccase	Unidentified marine fungi	Paper mill bleach plant effluent	Raghukumar et al., 1996
POX	*Coprinus cinereus*	Phenols	Kauffman et al., 1999
	Soybean	Chlorophenol	Caza et al., 1999;
		Phenol	Wright and Nicell, 1999;

			Kinsley and Nicell, 2002; Kennedy et al., 2002
	Horseradish	Phenols	Wagner and Nicell, 2002
	Turnip	Phenols	Duartz-Vazquez et al., 2002
	Coprinus cinereus	Phenols	Ikehata et al., 2003
	Horseradish	2,6-DCP	Laurenti et al., 2003
		Phenol	Wagner and Nicell, 2003; Entezari et al., 2004; Bodalo et al., 2006
	Bitter gourd	Phenol and their derivatives	Akhtar and Husain, 2006
POX	*Pleurotus ostreatus*	RBBR	Shin et al., 1997;
		Triarylmethane, heterocyclic, azo, polymeric dyes	Shin and Kim, 1998
	Horseradish	Remazol Blue, Cibacron Red	Bhunia et al., 2001
	Ipomea palmata, *Saccharum spontaneum*	Azo dyes	Shaffiqu et al., 2002
	Bacillus sp.	Textile effluents	Gudelj et al., 2002
MP-11	Horse heart cytochrome C	Synthetic dyes	Wariishi et al., 2002
POX	Bitter gourd	Reactive dyes	Akhtar et al., 2005a; 2005b
	Horseradish	Acid Black 10BX	Mohan et al., 2005
MnP	*Phanerochaete chrysosporium*	Azo dyes	Pasti-Grigsby et al., 1992
	Phanerochaete chrysosporium	Synthetic dyes	Rodriguez-Couto et al., 2002;
		Poly R-478	Mielgo et al., 2002;
		Sulfonaphthalein dyes	Christian et al., 2003;
		Crystal Violet, Poly R-478	Moldes et al., 2003
	Irpex lacteus	Dye effluent	Shin, 2004
	Lentinula edodes	Synthetic dyes	Boer et al., 2004
	Basidiomycete PV002	Ranocid fast Blue, Acid Black 210	Verma and Madamwar, 2005
	Basidiomycete fungus	RBBR	Machado et al., 2005
	Collybia dryophila	Synthetic dyes	Baldrian and Snajdr, 2006
	Ischnoderma resinosum	Textile dyes	Kokol et al., 2007

	WRF, strain L-25	Azo, diazo and anthraquinone	Reza et al., 2007
	Schizophyllum sp. F17	Azo dyes	Cheng et al., 2007
MnP+	*Pleurotus* sp.	OrangeG, RBBR	Eichlerova et al., 2006
Lac	10 fungal strains	Synthetic dyes	Park et al., 2007
LiP	*Phanerochaete chrysosporium*	Crystal Violet	Bumpus and Brock, 1988
		Azo, triphenylmethane and heterocyclic dyes	Cripps et al., 1990; Ollikka et al., 1993
	WRF	Synthetic dyes	Young and Yu, 1997
	Phanerochaete chrysosporium	GOG	Ollikka et al., 1998
		RBBR	Peralta-Zamora et al., 1999
		Synthetic dyes	Verma and Madamwar, 2002
	Pseudomonas desmolyticum	Direct Blue 6	Kalme et al., 2007
	Funalia trogii	Acid Black 52	Park et al., 2006
LiP + MnP	*Phanerochaete chrysosporium*	Reactive Brilliant Red	Yu et al., 2006
DyP	*Geotrichum candidum* Dec 1	Anthraquinone dyes	Kim and Shoda, 1999
	Geotrichum candidum	Xenobiotic compounds	Shoda, 2003; 2004
	Thanatephorus cucumeris	Azo and antraquinone dyes	Sato et al., 2004

thermostability (Sugano et al., 2004). Yang et al. (2003) have shown that the two yeasts, *Debaryomyces polymorphus*, *Candida tropicalis*, and two filamentous fungi, *Umbelopsis isabellina*, *Penicillium geastrivorus*, could completely decolorize 100 mg L^{-1} Reactive Black 5 within 16-48 h. MnP activity between 60 and 424 U L^{-1} were detected in culture supernatants of three of these organisms and it was observed that the color removal was due to enzymatic degradation of dyes. Extensive decolorization by *Debaryomyces polymorphus* (69-94%) and *Candida tropicalis* (30-97%) was obtained with five other azo dyes and one anthraquinone dye. Except for Reactive Brilliant Blue KNR and Reactive Yellow M-3R, the four azo dyes, Reactive Red M-3BE, Procion Scharlach H-E3G, Procion Marine H-EXL and Reactive Brilliant Red K-2BP, induced *Debaryomyces polymorphus* to produce MnP (105-587 U L^{-1}). However, MnP activity of 198-329 U L^{-1} was only found in the culture of *Candida tropicalis* containing Reactive Red M-3BE and Reactive Brilliant Red K-2BP, respectively.

The potential of a newly isolated fungus, *Geotrichum candidum* was investigated for the degradation of many xenobiotic compounds such as synthetic dyes, food coloring agents, molasses, organic halogens, lignin and kraft pulp effluents. The broad-spectrum degradation of these compounds is associated mainly with peroxidases produced by the fungus (Shoda, 2003; 2004). The dye-decolorizing peroxidase, DyP was a key enzyme in the decolorization and degradation of azo and anthraquinone dyes by fungus *Thanatephorus cucumeris* Dec 1 cells (Sato et al., 2004).

1.6.9. Application of redox mediators

Decolorization of dyes and pigments has been recently explored in the presence of redox mediators by some investigators (van der Zee et al., 2001; 2003). The use of redox mediators enhanced the electron transfer capacity and subsequent decoloration in anaerobic thermophillic bioreactors (Dos Santos et al., 2003; 2004; 2005). In almost all the studies, quinines were successfully used as redox mediators with special reference to AQDS in anaerobic biotransformation of azo dyes (Dos Santos et al., 2003). Quinones are the electron accepting moieties of humic substances and such compounds have been shown to play a vital role not only as a final electron acceptor for many recalcitrant

compounds, but also facilitates electron transfer from an electron donor to an electron acceptor e.g., azo dyes (Cervantes et al., 2001; Dos Santos et al., 2004). Therefore use of a redox mediator to accelerate the azo dye reduction and subsequent color removal from dye solutions/textile effluents is gaining importance as a promising technology.

Goodwin et al. (1996) found that many drugs, industrial pollutants, and other xenobiotics are known to be oxidized by peroxidases to potentially harmful free-radical intermediates. These investigators have shown that certain compounds, acting as efficient peroxidase substrates, may stimulate the formation of reactive free radicals by acting as mediators of electron transfer reactions, redox mediators. In order to understand this concept, the interaction of two well-known peroxidase substrates, chlorpromazine (CPZ) and aminopyrine (AP) has been studied. As shown by ESR and UV-visible spectroscopy, CPZ radical was able to oxidize AP to AP^+. Transient-state and steady state kinetic studies both exhibited that rate constants for CPZ oxidation to CPZ^+ by HRP were about 100-fold greater than for the corresponding HRP-catalyzed oxidation of AP to AP^+. When both AP and CPZ were present with HRP and H_2O_2, AP^+ formation was stimulated 100-fold. Concomitantly, the accumulation of CPZ^+ was completely inhibited in the presence of AP. Similar results were obtained when LPO, MPO, or the MPO mimic HOCl were substituted for HRP. This data suggested that CPZ worked as a redox mediator for peroxidase-catalyzed oxidation of AP and other chemicals. It was described that some peroxidase substrates, acted as redox mediators, may stimulate the production of toxic free-radical intermediates from various drugs and other xenobiotics. In another study, Goodwin et al. (1997) further found that CPZ can stimulate isoniazid oxidation as measured by nitroblue tetrazolium (NBT) reduction and O_2 consumption. The kinetics of isoniazid and CPZ oxidation by HRP in the presence of both compounds suggested CPZ was acting as an electron transfer mediator between HRP and isoniazid. Indeed, CPZ^+, the product of CPZ oxidation by HRP, was able to oxidize isoniazid. Thus, by acting as a redox mediator and preventing HRP inactivation, CPZ stimulated isoniazid oxidation by several orders of magnitude. Similarly, other efficient peroxidase substrates, such as phenol and tyrosine, were also able to dramatically stimulate isoniazid oxidation by HRP. They suggested that the presence of efficient peroxidase substrates might potentiate the oxidation of isoniazid and other hydrazines.

Olorunniji et al. (2000) have demonstrated the effect of 3,3′,5,5′-tetramethylbenzidine (TMB) on peroxidase-catalyzed oxidation of promethazine at pH 5.4. Increasing concentrations of TMB however, decreased the lag period proportional to the TMB concentration. Addition of promethazine to preformed charge transfer complex caused rapid bleaching of the blue-colored complex. Titration of promethazine with the yellow-colored diimine gave rise to the blue charge-transfer complex and the complete reduction of the species to the colorless parent amine compound. The available evidence showed that promethazine was oxidized via redox mediation by TMB peroxidase-oxidized products.

Oxidative degradation of cis-and trans-1,4-polyisoprenes by two types of enzyme-mediator systems, lipoxygenase/linoleic acid and HRP/HOBT, was investigated at 37 °C in aqueous media and analyzed by gel permeation chromatography. Lipoxygenase and HRP activated their substrates, linoleic acid and HOBT, respectively, for scission of main chains of both 1,4-polyisoprenes. M_r of 1,4-polyisoprenes decreased during the treatment under both enzyme-mediator systems (Enoki et al., 2003a). Enoki et al. (2003b) further shown the oxidative degradation of trans-1,4-polyisoprene cast films and single crystals by enzyme-mediator systems. Oxidative degradation of cis- and trans-1,4-polybutadienes by HRP/HOBT was also carried out by the same group (Enoki et al., 2004). Chromium and manganese half-sandwich complexes were evaluated as mediators to glucose oxidase since they are of similar size to ferrocene derivatives (sandwich complexes) and contain a single pi-ligand for interaction with the enzyme co-factor. The activity of HRP and other enzymes was also mediated by similar complex (Forrow and Walters, 2004). Won et al. (2004) have described the HRP-catalyzed polymerization of cardanol in aqueous organic solvent in the presence of a redox mediator. Violuric acid (VLA), N-ethyl phenothiazine (NEPT), and PTPA were tested as a mediator. It was surprising that the HRP-catalyzed polymerization of cardanol took place in the presence of NEPT or PTPA.

Karaseva et al. (2002) demonstrated a comparative study on the kinetics of peroxidase-catalyzed oxidation of TMB in the presence of 2,4-dinitrosoresorcinol (DNR), its polydisulfide derivative [poly(DNRDS)], and resorcinol polydisulfide [poly(RDS)], substances that competitively inhibit the formation of TMB conversion product, was carried out. The kinetic parameters of poly(RDS) suggested that it was the most efficient

inhibitor of peroxidase oxidation of TMB: in μM concentrations, it completely stopped this process and could be used in EIA. Furthermore, same workers reported that peroxidase-catalyzed oxidation of ABTS and TMB was activated by tetrazole and its 5-substituted derivatives-5-amino-(AmT), 5-methyl- (MeT), 5-phenyl- (PhT), and 5-CF3-(CF3-T) tetrazoles. In phosphate-citrate or phosphate buffer (pH 6.4 or 7.2; 20 °C), the activating effect of tetrazoles on TMB and ABTS oxidation decreased in the series AmT > MeT > T > PhT > CF3-T and T > AmT > MeT > PhT, respectively. Such findings suggested that AmT, MeT, and T could be used as activators of peroxidase-catalyzed oxidation of TMB and ABTS, as well as in designing peroxidase-based biosensors (Karaseva et al., 2005).

The tuberculostatic drug rifampicin has been described as a scavenger of reactive species. Additionally, the recent demonstration that oral therapy with a complex of rifampicin and HRP was more effective than rifampicin alone, in an animal model of experimental leprosy, suggested the importance of redox reactions involving rifampicin and their relevance to the mechanism of action. Hence it has been shown that the oxidation of rifampicin catalyzed by HRP, since this enzyme may represent the prototype of peroxidation-mediated reactions. It was found that the antibiotic was effectively oxidized and rifampicin-quinone was the product, in a reaction dependent on both HRP and H_2O_2 (dos Santos Fde et al., 2005).

Akhtar et al. (2005a) have reported the decolorization of 21 different reactive textile and other industrially important dyes by using BGP in sodium acetate buffer, pH 5.6 in the presence of 0.75 mM H_2O_2 and 1.0 mM HOBT. Decolorization rate was drastically increased when dyes were treated with BGP in the presence of 1.0 mM HOBT. Treating with BGP in the presence of 1.0 mM HOBT also significantly decolorized polluted wastewater containing complex mixtures of dyes. In a recent study, Matto and Husain (2007) demonstrated the effect of various redox mediators on the salt fractionated turnip (*Brassica rapa*) proteins mediated decolorization of direct dyes, used in textile industry. The rate and extent of decolorization of dyes was significantly enhanced by the presence of different types of redox mediators. Six out of ten investigated compounds have shown their potential in enhancing the decolorization of direct dyes. The performance was evaluated at different concentrations of mediator and

enzyme. The efficiency of each natural mediator depends on the type of dye treated. The decolorization of all tested direct dyes was maximum in the presence of 0.6 mM redox mediator at pH 5.5 and 30 °C. Complex mixtures of dyes were also maximally decolorized in the presence of 0.6 mM redox mediator (HOBT/VLA). Total organic carbon analysis of treated dyes or their mixtures showed that these results were quite comparable to the loss of color from solutions.

MnP H 5 from the WRF, *Phanerochaete chrysosporium*, in the presence of either Mn(II) (10 mM) or GSH (10 mM) was able to mineralize ^{14}C-U-ring-labeled 2-amino-4,6-dinitrotoluene up to 29% in 12 d. The mineralization extent reached 82% in the presence of both Mn(II) and GSH. However, there was no significant mineralization observed in the absence of both Mn(II) and GSH. However, these findings suggested the involvement of a mediator [either Mn(II) or GSH] in the degradation of 2-amino-4,6-dinitrotoluene by MnP. Using ESR techniques, it was found that the glutathionyl free radical (GS*) was produced through the oxidation of GSH by MnP in the presence as well as in the absence of Mn(II). GS* was also generated through the direct oxidation of GSH by Mn(III). In this work an involvement of GS* in the GSH-mediated mineralization of 2-amino-4,6-dinitrotoluene by MnP has been described (Aken et al., 2000).

VA (3, 4-dimethoxy benzyl alcohol) is a secondary metabolite of several WRF. LiP catalyzed the oxidation to the VA^+ and this radical acted as a mediator for the degradation of lignin (Farrell et al., 1989). Mediating properties of VA could be enhanced if the radical is somehow complexed to the LiP (Lundell, 1993). It has been demonstrated that the oxidation of 4-methoxy mandelic acid (MMA) was mediated by VA. Increasing VA concentration in the presence of 2.0 mM MMA resulted in increased oxidation of MMA yielding anisaldehyde (Sutherland et al., 1995). It was discovered that LiP isozyme H 2 has the ability to oxidize Mn^{2+} (Khindaria et al., 1995). Koduri and Tien (1995) have investigated the LiP-catalyzed oxidation of guaiacol and the role of VA in this reaction by steady state and pre-steady-state methods. Chung and Aust (1995) investigated the indirect oxidation of PCP by LiP using VA as a mediator. PCP inhibited the oxidation of VA to veratryl aldehyde by LiP. Inhibition was characterized by lag period followed by the same rate of VA oxidation. Increasing concentration of PCP increased the lag period

before VA oxidation. During the lag period, PCP was oxidized and the extent of PCP oxidation increased with increasing concentration of VA. VA stimulates LiP probably by protecting the enzyme against the damaging effect of H_2O_2 (Akhtar et al., 1997). Some workers have investigated the role of fungal metabolite 2-chloro-1,4-dimethoxybenzene (2Cl-1,4-DMB) in the oxidation of Poly R 478, MMA and oxalic acid by LiP. It was noticed that 2Cl-1,4-DMB can replace the function of VA as a redox mediator in LiP-catalyzed oxidations (Teunissen and Field, 1998a). These workers have further reported that 2Cl-1,4-DMB oxidation by LiP proceeds via the formation of the 2Cl-1,4-DMB cation radical as indicated by ESR and UV/vis spectroscopy (Teunissen and Field, 1998b). LiP from *Bjerkandera adusta* showed low activity in the transformation of six industrial azo and phthalocyanine dyes, but the specific activity increased 8 to 100 fold when VA was included in the reaction mixture (Heinfling et al., 1998b). The mechanism of the VA-mediated oxidation of isoeugenyl acetate (IEA) by LiP, and the subsequent spontaneous C-α-C-β cleavage of IEA to vanillyl acetate were studied. IEA oxidation only occurred in the presence of VA (ten Have et al., 1999).

Maximum decolorization of dyes; Procion Brilliant Blue HGR, Ranocid Fast Blue, Acid Red 119 and Navidol Fast Black MSRL with *Phanerochaete chrysosporium* partially purified LiP, was observed at 0.2 and 0.4 mM H_2O_2, 2.5 mM VA and pH 5.0 after 1 h reaction, using 50 ppm of dyes and 9.96 mkat L^{-1} of enzyme (Verma and Madamwar, 2002). Baciocchi et al. (2002) described the role of VA as a mediator in the oxidative biodegradation of lignin by LiP. Huang et al. (2003) have shown that VA at higher concentration stimulated the LiP-catalyzed oxidation of phenolic compounds remarkably. This novel phenomenon was due to its competition with the phenols for the active site of the enzyme and to the high reactivity of the formed VA^+ radical, which resulted in an additional oxidation of the phenols. LiP and MnP carried out direct and indirect oxidation as well as reduction of xenobiotic compounds. Indirect reactions involved redox mediators such as VA and Mn^{2+} (Christian et al., 2005). Bioelectrocatalytic reduction of H_2O_2 was studied by LiP-modified graphite electrodes to elucidate the ability of LiP to electro-enzymatically oxidize phenols, catechols, as well as VA and some other high-redox-potential lignin model compounds. The bioelectrocatalytic reduction of H_2O_2 mediated by VA and effects of VA on the

efficiency of bioelectrocatalytic oxidation of other co-substrates acting as mediators were investigated. The bioelectrocatalytic oxidation of phenol- and catechol derivatives and ABTS by LiP was independent of the presence of VA, whereas the efficiency of the LiP bioelectrocatalysis with the majority of other LMC acting as mediators increased upon addition of VA. Special cases were phenol and MMA. The obtained results have demonstrated different mechanisms for the bioelectrocatalysis of LiP depending on the chemical nature of the mediators and are of a special interest both for fundamental science and for application of LiP in biotechnological processes (Ferapontova et al., 2006).

1.7. APPLICATIONS OF IMMOBILIZED ENZYMES

Several limitations prevent the use of free oxidoreductases in the decolorization and degradation of dyes such as the stability and catalytic ability of enzymes decrease with the complexity of the effluents (Zille et al., 2003). Some of these limitations have been overcome by the use of enzymes in the immobilized form, which could be used as catalysts with longer lifetime (Rogalski et al., 1995). In industrial applications immobilization of enzymes would allow the reuse of the enzyme and thus reduce the cost for such processes (Akhtar et al., 2005c; Khan et al., 2005). Some applications of immobilized peroxidases in the removal of organopollutants are shown in Table 2.

HRP immobilized carbon electrode was capable of catalyzing the oxidation and detoxification of 44 μM TNT in aqueous solutions under optimized conditions. The removal rate of TNT for the electro-enzymatic method was much greater than for electrochemical and biochemical methods. Stoichiometric and kinetic studies indicated that the H_2O_2 was utilized more effectively in the electro-enzymatic method. Denitrification as intermediate reaction was also investigated (Lee et al., 2003).

Tatsumi et al. (1996) immobilized HRP on magnetite for the removal of chlorophenols. It was found that HRP was selectively adsorbed on magnetite and each chlorophenol was removed almost 100%. The effect of HRP immobilized on activated alumina on the removal efficiency of phenol showed that one molecule of HRP was needed to remove approximately 1100 molecules of phenol when the reaction was

conducted at pH 8.0 and room temperature (Vasudevan and Li, 1996). Singh and Singh (2002) have reported the development of an enzymatic method for the removal of phenols from industrial wastewater, using TP. Peroxidase, covalently bound to silica, showed 95% removal of phenol, whereas naphthol was removed up to 99%. However, calcium alginate entrapped TP and polyacrylamide gel entrapped TP removed 50% and 60.4 % of phenol, respectively. Regalado et al. (2004) have reported the removal of more than 90% of phenol after 3 h of reaction using alginate entrapped TP. The immobilized HRP was used for the removal of *p*-chlorophenol. The polymerization of *p*-chlorophenol into insoluble precipitate was completed within 3 h after the initiation of reaction with the addition of H_2O_2 with a maximal removal efficiency of 25% (Lai and Lin, 2005).

In a more recent study, the model wastewater contaminated with phenols was treated with soluble and immobilized BGP. Maximum removal of phenols was found in the buffers of pH 5.0-6.0 and at 40 °C in the presence of 0.75 mM H_2O_2. Significantly higher level of TOC was removed from the model wastewater containing individual phenol and mixture of phenols by immobilized BGP as compared to the soluble enzyme (Akhtar and Husain, 2006). HRP was successfully immobilized on aluminum-pillared interlayered clay (Al-PILC) to obtain enzyme-clay complex for the treatment of wastewater polluted with phenolic compounds. The immobilized HRP exerted a perfect phenol removal by precipitation or transforming to other products over a broader pH range from 4.5 to 9.3 (Cheng et al., 2006). SBP and HRP were immobilized on glutaraldehyde-activated aminopropyl glass beads (Gomez et al., 2006). The activities of the immobilized SBP and HRP were 74% and 78%, respectively, of the corresponding free enzymes, which, together with their high protein content, made them suitable catalysts for the enzymatic elimination of phenol from aqueous solutions in the presence of H_2O_2. The reaction with immobilized HRP was faster than SBP at all the enzyme concentrations assayed. However, at enzyme concentrations lower than 0.028 mg mL^{-1}, 7% and 20% more phenol was removed with SBP than with HRP. This behavior changed as the enzyme dose increased. In a recent study, Gomez et al. (2007) immobilized SBP by covalently binding to glass supports with different surface areas and these immobilized preparations were used in a laboratory scale fluidized bed reactor to study their viability for use in phenol removal. The influence of the different operational variables on the

process was also studied. When derivatives immobilized on supports with the highest surface area were used, 80% removal was achieved.

Zhang et al. (1999) designed reactors of three different configurations, continuous packed-bed, fed-batch fluidized-bed, and continuous fluidized-bed bioreactor and used for the decolorization of Orange II, by MnP and LiP of WRF. The degradation of Orange II in a continuous packed-bed bioreactor for periods longer than 30 d was carried out by immobilized *Phanerochaete chrysosporium* containing MnP (Mielgo et al., 2001). Nearly complete decolorization was achieved when working at a high dye load rate (0.2 g $L^{-1}d^{-1}$) at 37 °C and applying oxygen gas in a pulsed flow.

Fruhwirth et al. (2002) have used a catalase peroxidase (CP) from the newly isolated *Bacillus* SF to treat textile-bleaching effluents. The enzyme was immobilized on various alumina-based carrier materials with different shapes. Bleaching effluent was treated in a horizontal packed-bed reactor containing 10 kg of the immobilized CP at a textile-finishing company. The treated liquid (500 L) was reused within the company for dyeing fabrics with various dyes, resulting in acceptable colour differences of below Delta E*=1.0 for all dyes. Shaffiqu et al. (2002) used hydrophobic matrix bound *Saccharum* peroxidase for the degradation of four textile dyes; Procion Navy Blue HER, Procion Brilliant Blue H-7G, Procion Green HE-4 BD, and Supranol Green. These dyes at an initial concentration of 50 mg L^{-1} were completely degraded within 8 h by the enzyme immobilized on the modified polyethylene matrix. The immobilized enzyme was used in a batch reactor for the degradation of Procion Green HE-4BD and the reusability was studied for 15 cycles and the half-life was found to be 60 h. Silica gel immobilized microperoxidase 11 was quite successful in decolorizing a water insoluble dye; normally this dye is used in leather industry (Kadnikova and Kostic, 2003).

Performance of free HRP verses immobilized HRP (alginate and acrylamide polymeric matrix) was evaluated in the process of dye removal in order to assess the reusability of HRP. Acrylamide gel immobilized HRP showed effective performance compared to free HRP and alginate entrapped HRP. Alginate entrapped HRP showed inferior performance over the free enzyme due to the consequence of non-availability of the enzyme to the dye molecule due to polymeric immobilization (Mohan et al., 2005). BGP immobilized on a bioaffinity support, Con A Sephadex was highly effective in

decolorizing industrially important dyes from polluted water compared to its soluble counterpart. The dye decolorization was maximum in the buffer of pH 3.0 and at 40 °C. This enzyme was repeatedly exploited for the decolorization of eight reactive textile dyes from fresh batch of dye solutions and after 10th repeated use the immobilized enzyme still retained nearly 50% of the initial enzyme activity. The mixtures of dyes were decolorized by more than 80% when treated with immobilized BGP. Immobilized BGP was capable of removing remarkably very high percentage of color from the dyeing effluent. TOC content of soluble and immobilized BGP treated individual dyes, mixture of dyes and dyeing effluent was determined and it was observed that higher TOC was removed after treatment with immobilized BGP (Akhtar et al., 2005b).

Combining enzymatic catalysis and the electrochemical generation of H_2O_2, an electro-enzymatic process was developed by Kim et al. (2005). In this study, an electroenzymatic method that uses an immobilized HRP was investigated to degrade Orange II within a two-compartment packed-bed flow reactor. To evaluate the electroenzymatic degradation of Orange II, electrolytic experiments were carried out with 0.42 U mL^{-1} HRP at −0.5 V. The overall application of the electroenzymatic led to a greater degradation rate than the use of electrolysis alone.

Bromophenol Blue and Methyl Orange removal capabilities of citraconic anhydride-modified HRP were compared with those of native HRP. Upon the chemical modification, the decolorization efficiencies were increased by 1.8% and 12.4% for Bromophenol Blue and Methyl Orange, respectively. Experimental data revealed that aqueous phase pH, reaction time, temperature, enzyme concentration and ratio of dye and H_2O_2 play a significant role on the dye degradation. Lower dose of citraconic anhydride-modified HRP was required than that of native enzyme for the decolorization of both dyes to obtain the same decolorization efficiencies. Citraconic anhydride-modified HRP exhibited a good decolorization of dye over a wide range of dye concentration from 8 to 24 or 32 μmol L^{-1} at 300 μmol L^{-1} H_2O_2, which would match industrial expectations. Kinetic constants for two different dyes were also determined (Liu et al., 2006).

Table 2: Applications of immobilized peroxidases in the removal of organopollutants

Enzyme	Source	Support	Applications	References
Peroxidase	Horseradish	Carbon electrode	TNT	Lee et al., 2003
Peroxidase	Horseradish	Magnetite	Chlorophenols	Tatsumi et al., 1996
		Alumina	Phenol	Vasudevan and Li, 1996
			p-chlorophenol	Lai and Lin, 2005
		Aluminum-pillared interlayered clay	Phenols	Cheng et al., 2006
	Turnip	Silica, calcium alginate, polyacrylamide gel	Phenol, naphthol	Singh and Singh, 2002; Regalado et al., 2004
	Bitter gourd	Con A-Sephadex	Phenols	Akhtar and Husain, 2006
	Horseradish + soybean	Glass beads	Phenols	Gomez et al., 2006
	Soybean	Glass support	Phenol	Gomez et al., 2007
Catalase-peroxidase	*Bacillus SF*	Various alumina based supports	Textile bleaching effluent	Fruhwirth et al., 2002
Peroxidase	*Saccharum uvarum*	Hydrophobic support (modified polyethylene)	Procion Navy Blue HER, Procion Brilliant Blue H-7G, Procion Green HE-4BD, and Supranol Green	Shaffiqu et al., 2002
Micro-peroxidase 11	Undecapeptide from horse heart cytochrome C	Silica gel	Water insoluble synthetic dyes	Kadnikova and Kostic, 2003
Peroxidase	Bitter gourd	Con A-Sephadex	Reactive textile dyes	Akhtar et al., 2005b
	Horseradish	Polyacrylamide gel	Acid Black 10BX	Mohan et al., 2005
		Citraconic-anhydride	Bromophenol Blue and Methyl Orange	Liu et al., 2006

OBJECTIVES OF THE PRESENT WORK

- Peroxidases perform a variety of biological functions, such as the synthesis of biomolecules and the detoxification of H_2O_2. Recently, it has been found that peroxidases have significant role in the detoxification and degradation of aromatic pollutants. In view of their wide spectrum applications here peroxidases from three different plant sources, bitter gourd (*Momordica charantia*), turnip (*Brassica rapa*) and tomato (*Lycopersicon esculentum*) have been selected for this investigation.
- In the first part (Chapter II) of the study, we have investigated the immobilization of partially purified BGP on DEAE cellulose for its inexpensive, simple and high yield immobilization followed by stabilization against several forms of inactivation.
- In the second part (Chapter III) of the study, an attempt has been made to cut down the cost of immobilized enzyme preparation. Jack bean extract was used as a source of Con A for the preparation of Con A-cellulose support. This support has been selected for the immobilization of ammonium sulphate fractionated turnip proteins containing peroxidases. The stability of Con A-cellulose bound TP was examined against various parameters like pH, heat, urea, guanidinium-HCl, detergents and water-miscible organic solvents.
- In the third part of the work (Chapter IV) the soluble TP has been employed in the presence of a redox mediator, HOBT, for the decolorization of five acid dyes with wide spectrum chemical groups. Various experimental conditions have been standardized for the treatment of acid dyes and their mixtures by TP.
- In the last part (Chapter V) of this study an effort has been made to investigate the potential of Con A-cellulose immobilized turnip and tomato peroxidases to decolorize textile carpet industrial effluents. Various experimental conditions have been optimized for the treatment of textile effluents by immobilized TP/TMP. The effluent disappearance was monitored by using UV-visible absorbance spectrophotometry and TOC analyzer.

CHAPTER-II

Direct Immobilization of Peroxidase on DEAE Cellulose from Ammonium Sulphate Fractionated Proteins of Bitter Gourd (*Momordica charantia*)

2.1. INTRODUCTION

Recently, it has been reported that peroxidases can be used in the detoxification and biotransformation of several phenols, aromatic amines, biphenyls, bisphenols and dyes present in polluted wastewater/industrial effluents coming out from several industries (Husain and Jan, 2000; Duran and Esposito, 2000; Bhunia et al., 2001; Duran et al., 2002; Torres et al., 2003). The soluble enzyme cannot be exploited at large scale due to some inherent limitations to treat the huge volume of effluents. On the other hand, the immobilized enzyme has offered several advantages, such as enhanced stability, easier product recovery and purification, protection of enzymes against denaturants, proteolysis and reduced susceptibility to contamination. Several methods have been used for the immobilization of peroxidases from various sources but most of the immobilized enzyme preparations either use commercially available enzyme or expensive supports (Husain et al., 1992; Akhtar et al., 2005c). These expensive immobilized systems could not meet the requirements for the treatment of bulk of industrial effluents. However, among the techniques used for the immobilization of enzymes, adsorption on the insoluble supports has several merits over the other known methods.

Adsorption procedures are significantly useful for the immobilization of enzymes directly from the crude homogenate and thus avoiding the high cost of enzyme purification (Batra and Gupta, 1994b; Batra et al., 1997). Ease of immobilization, lack of chemical modification and usually accompanying enhancement in stability are some of the advantages offered by the adsorption procedures. In order to minimize the cost of immobilization, the adsorption of proteins directly from partially purified enzyme preparation on an anion exchanger can be performed. These supports have already appeared in high yield and stable immobilization of enzymes (Reddy et al., 2004).

In this work, an effort has been made to immobilize peroxidases on an anion exchanger, DEAE (diethyl aminoethyl) cellulose directly from the dialyzed solution of salt fractionated proteins of bitter gourd. DEAE cellulose adsorbed BGP was compared to its soluble counterpart for its stability against pH, heat, urea, detergents, water-miscible organic solvents and proteolytic enzyme, trypsin.

2.2. MATERIALS AND METHODS

2.2.1. Materials

DEAE cellulose 11 was the product of SRL chemicals, Mumbai, India. *o*-dianisidine-HCl was purchased from IGIB, New Delhi, India. Ammonium sulphate, dioxane, dimethyl sulphoxide, *n*-propanol and Triton X-100 were obtained from the SRL Chemicals, Mumbai, India. Surf Excel and Rin Powder were purchased from local market. Other chemicals and reagents employed were of analytical grade and were used without any further purification.

2.2.2. Ammonium sulphate fractionation of bitter gourd proteins

Bitter gourd (50 g) was homogenized in 100 mL of 50 mM sodium acetate buffer, pH 5.6. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000 x *g* on a Remi Cooling Centrifuge R-24 for 10 min at 4 °C. The clear supernatant was subjected to salt fractionation by adding 20-80% (w/v) $(NH_4)_2SO_4$. The solution was stirred overnight at 4 °C and the obtained precipitate was collected by centrifugation at 10,000 x *g* on a Remi Cooling Centrifuge R-24 for 10 min at 4 °C (Akhtar et al., 2005c). The collected precipitate was redissolved in 50 mM sodium acetate buffer, pH 5.6 and dialyzed against the assay buffer.

2.2.3. Activation of DEAE cellulose

DEAE cellulose (5.0 gm) was added to 100 mL of distilled water and was stirred slowly, overnight for swelling. Swelled DEAE cellulose was filtered on a Buchner funnel and was incubated with 100 mL of 0.5 N HCl for 1 h. Acid treated anion exchanger was collected by filtration on Buchner funnel and was washed with distilled water continuously till it attained pH 7.0. Hundred milliliters of 0.5 N NaOH was added to HCl treated DEAE cellulose and the slurry was stirred on a magnetic stirrer for 1 h at room temperature, 25 °C. Treated anion exchanger was washed again with distilled water till it attained neutral pH. Further, it was stored in 100 mL of distilled water at 4 °C.

2.2.4. Adsorption of BGP on activated DEAE cellulose

BGP (5535 U) was added to 5.0 g of activated DEAE cellulose and stirred in 50 mM sodium acetate buffer, pH 5.6, at 4 °C overnight. Unbound BGP was removed by extensive washing with assay buffer.

2.2.5. Effect of ion concentrations on the DEAE cellulose adsorbed BGP

The adsorbed BGP preparation (1.25 U) was incubated with increasing concentrations of NaCl (0.1-1.0 M) in 50 mM sodium acetate buffer, pH 5.6 for 1 h at 37 °C. In order to monitor the effect of long time exposure of immobilized enzyme with ions, the adsorbed BGP was also incubated with 0.1 M NaCl upto 24 h.

2.2.6. Effect of trypsin mediated proteolysis on soluble and immobilized BGP

Soluble and immobilized BGP (1.25 U) were incubated with 0.25-2.5 mg mL^{-1} of trypsin at 37 °C for 1 h (Akhtar et al., 2005c). The activity of soluble and immobilized BGP in assay buffer without any trypsin treatment was taken as control (100%), for the calculation of remaining percent activity. Peroxidase activity was determined according to the standard procedure.

2.2.7. Effect of Surf Excel and Rin Powder on soluble and immobilized BGP

Soluble and immobilized BGP (1.25 U) were incubated independently with varying concentrations of Surf Excel and Rin Powder (0.1-1.0%, w/v) in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated detergent concentrations and other assay conditions were same as described in the text. The activity of soluble and immobilized BGP in assay buffer without any detergent was taken as control (100%), for the calculation of remaining percent activity.

2.2.8. Effect of Triton X-100 on soluble and immobilized BGP

Soluble and immobilized BGP (1.25 U) were incubated with increasing concentrations of Triton X-100 (0.5-5%, v/v) in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was determined at all the indicated detergent concentrations after the incubation period.

2.2.9. Effect of water-miscible organic solvents on soluble and immobilized BGP

Soluble and immobilized BGP (1.25 U) were incubated with 10-60% (v/v) of water-miscible organic solvents; dioxane/DMSO (dimethyl sulphoxide)/*n*-propanol in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was determined at all the indicated organic solvent concentrations after the incubation period.

2.2.10. Measurement of peroxidase activity

Peroxidase activity was determined from a change in the optical density (A_{460} nm) by measuring the initial rate of oxidation of 6.0 mM *o*-dianisidine-HCl in the presence of 18 mM H_2O_2 in 50 mM sodium acetate buffer, pH 5.6 for 15 min at 37 °C.

The immobilized preparation was continuously agitated for the entire duration of assay. The assay was highly reproducible with immobilized preparation (Akhtar et al., 2005c).

One unit of peroxidase activity (U) was defined as the amount of enzyme protein that catalyzes the oxidation of 1 μmol of *o*-dianisidine-HCl per min at 37 °C into colored product (ε_m = 30,000 $M^{-1} L^{-1}$).

2.2.11. Determination of protein concentration

Protein concentration was estimated by the method of Lowry et al. (1951). A suitable aliquot of the protein sample was diluted to 1.0 mL with distilled water. To this,

5.0 mL of freshly prepared alkaline copper reagent was added. The alkaline copper reagent was prepared by mixing copper sulphate (1%, w/v), sodium potassium tartarate (2%, w/v) and sodium carbonate in 0.1 N NaOH in the ratio of 1:1:100. After 10 min incubation at room temperature, 0.5 mL of 1.0 N Folin's reagent was added. The contents were mixed and color intensity was read after 30 min against the reagent blank at 660 nm. The concentration of protein in the samples was determined using BSA as standard.

2.3. RESULTS

2.3.1. Adsorption of BGP on DEAE cellulose

DEAE cellulose is commonly used for the purification and immobilization of variety of enzymes and proteins. In view of DEAE cellulose property to adsorb proteins on the basis of ionic interactions, this property has been exploited for directly binding the enzymes from the dialyzed salt fractionated bitter gourd proteins by simply incubating DEAE cellulose with ammonium sulphate fractionated dialyzed proteins overnight at 4 °C. After adsorption of proteins on DEAE cellulose, the complex was washed with assay buffer till the traces of unbound proteins were removed. Unbound proteins were removed by extensive washing with assay buffer. DEAE cellulose adsorbed peroxidase activity 590 U g^{-1} of the matrix. The effectiveness factor 'η' of the immobilized enzyme preparation was 0.95 (Table 3). High effectiveness factor of immobilized BGP suggested that immobilized preparation was quite porous and effective in catalysis.

2.3.2. Effect of ion concentrations on the DEAE cellulose adsorbed BGP activity

The wastewaters may also contain several types of ions, therefore it was necessary to examine the detachment of BGP from its support in the presence of various concentrations of ions. The exposure of adsorbed enzyme with increasing concentrations of NaCl (0.1-1.0 M) for 1 h exhibited retention of very high enzyme activity even in the

Table 3: Adsorption of BGP on DEAE cellulose

Amount of enzyme activity loaded (X) (U)	Amount of enzyme activity in washes (Y) (U)	Activity bound g^{-1} of DEAE cellulose (U)			Activity yield (%) (B/Ax100)
		Theoretical (X-Y=A) (A)	Actual (B)	Effectiveness factor (η) (B/A)	
1107	482	625	590	0.95	95

Peroxidase activity was assayed according to the procedure given in the text. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation < 5%.

presence of 1.0 M NaCl. The incubation of immobilized BGP upto 0.5 M NaCl for 1 h had no detachment of enzyme activity (Fig. 1). In order to monitor the effect of long time exposure of immobilized enzyme with ions, the adsorbed BGP was incubated with 0.1 M NaCl upto 24 h. The incubation of immobilized enzyme with 0.1 M NaCl for 24 h resulted in a slight loss of 13% of the initial activity (Fig. 2).

2.3.3. Stability properties of soluble and DEAE cellulose bound BGP preparation

The stability of soluble and DEAE cellulose bound BGP was monitored against various physical and chemical parameters because these parameters can affect the activity of the enzymes used for the treatment of organic pollutants present in the wastewater.

2.3.4. pH-activity profiles of soluble and immobilized BGP

DEAE cellulose bound BGP showed broadening in the pH-activity profile as compared to the native enzyme (Fig. 3). Immobilized enzyme retained significantly higher enzyme activity on both sides of pH-optimum in comparison to free enzyme. pH-optimum of immobilized enzyme had no difference from pH 5.0 to 6.0, although soluble enzyme showed pH-optimum at pH 5.0.

2.3.5. Temperature-activity profiles of soluble and immobilized BGP

DEAE cellulose bound BGP preparation had no change in temperature-optima as compared to its soluble counterpart. Both preparations exhibited same temperature-optima at 40 °C. However, DEAE cellulose bound BGP retained significantly greater fractions of catalytic activity at higher temperatures (Fig. 4).

2.3.6. Thermal denaturation of soluble and immobilized BGP

Figure 5 demonstrates thermal denaturation of soluble and immobilized BGP at 60 °C for 2 h. Soluble BGP incubated at 60 °C for 2 h retained 43% of its initial enzyme

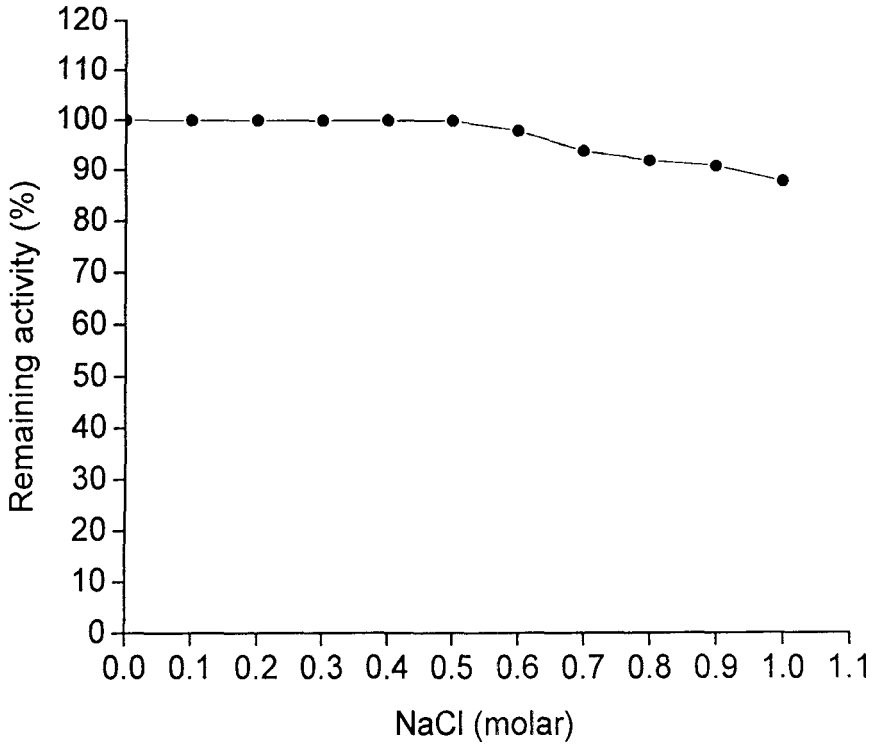

Figure 1: Effect of NaCl concentrations on the DEAE cellulose adsorbed BGP

DEAE cellulose adsorbed BGP (1.25 U) was incubated with increasing concentrations of NaCl (0.1-1.0 M) in 50 mM sodium acetate buffer, pH 5.6 for 1 h at 37 °C. After incubation period each treated enzyme preparation was centrifuged to monitor the remaining adsorbed enzyme activity. Symbol indicates immobilized (•) BGP.

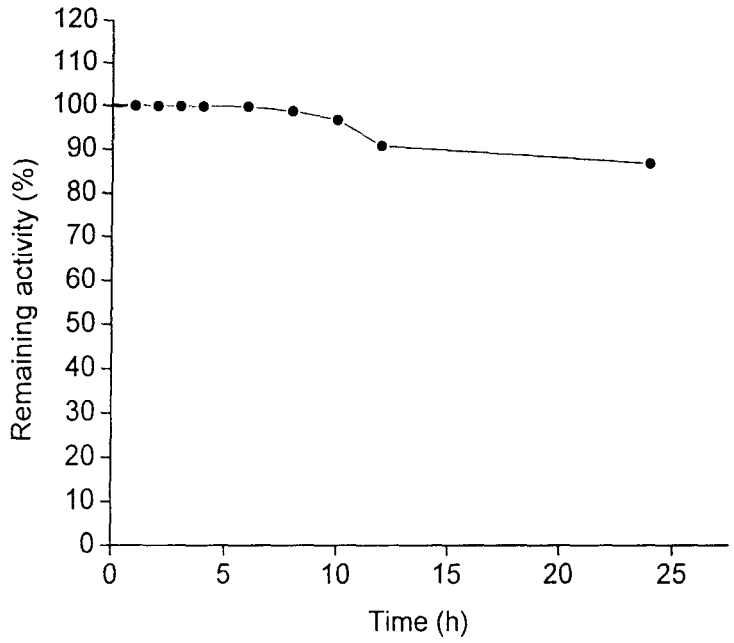

Figure 2: Effect of long time exposure of DEAE cellulose adsorbed BGP with ions

DEAE cellulose adsorbed BGP (1.25 U) was incubated with 0.1 M NaCl in 50 mM sodium acetate buffer, pH 5.6 upto 24 h at 37 °C. After indicated time of incubation each treated enzyme preparation was centrifuged to monitor the remaining adsorbed enzyme activity. Symbol indicates immobilized (•) BGP.

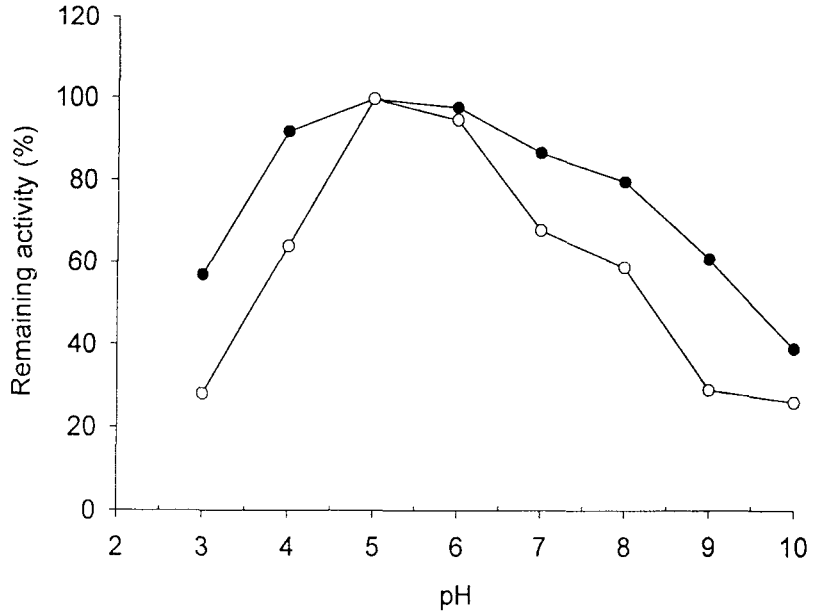

Figure 3: pH-activity profiles of soluble and immobilized BGP

The appropriate and equal amount of soluble and immobilized BGP was taken for monitoring the pH-activity profiles. The reaction mixture was incubated at 37 °C for 15 min in buffers of pH ranges from 3.0-10.0. The buffers used were glycine-HCl for pH 3.0, sodium acetate for pH 4.0, 5.0, sodium phosphate for pH 6.0-8.0 and Tris-HCl for pH 9.0 and 10.0. The molarity of each buffer was 50 mM. Symbols indicate, the soluble (o) and immobilized (•) BGP.

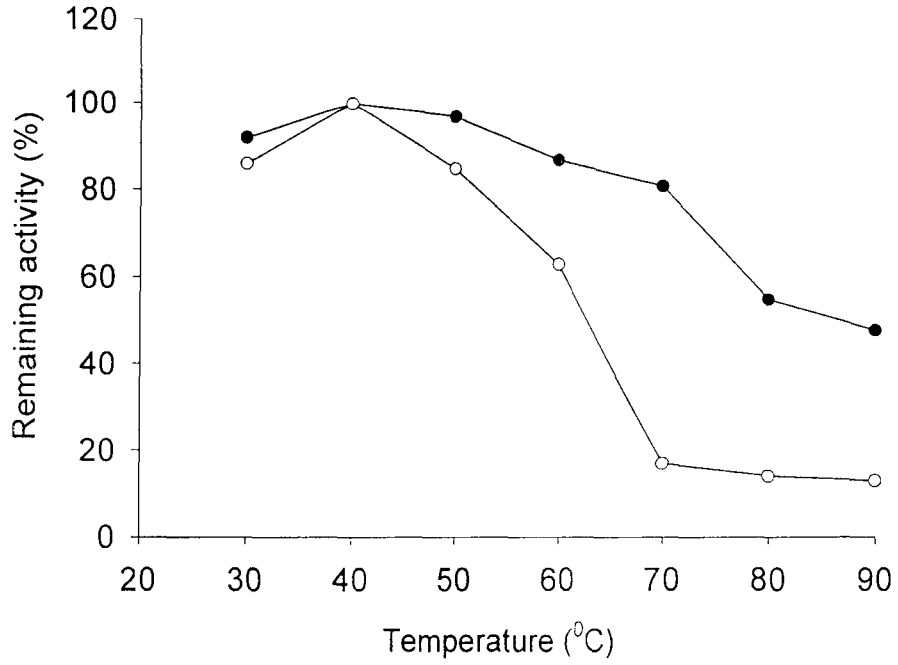

Figure 4: Temperature-activity profiles of soluble and immobilized BGP

The activity of an appropriate amount of soluble and immobilized BGP was monitored at various indicated temperatures. Activity expressed at 40 °C was taken as control (100%) for the calculation of remaining percent activity. Symbols indicate, the soluble (o) and immobilized (•) BGP.

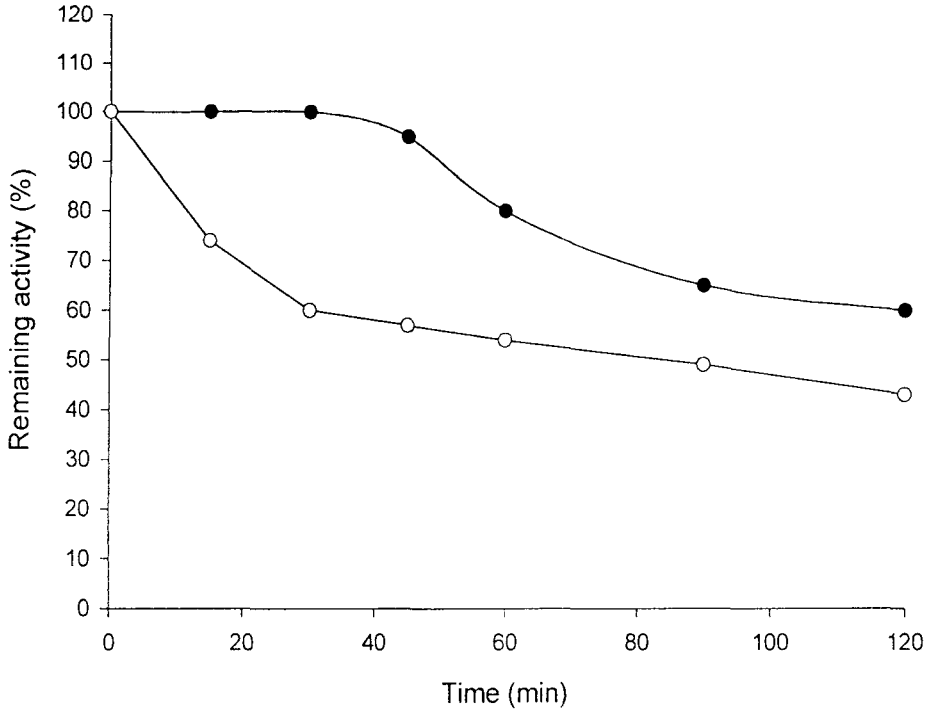

Figure 5: Thermal denaturation of soluble and immobilized BGP

Soluble and immobilized BGP (1.25 U) were incubated at 60 °C for varying times in 50 mM sodium acetate buffer, pH 5.6. Aliquots of each preparation were taken out at indicated time intervals and chilled quickly in crushed ice for 5 min. After chilling, all the tubes were brought to room temperature and activity was determined as described in the text. Un-incubated samples at 60 °C were taken as control (100%) for the calculation of remaining percent activity. Symbols indicate, the soluble (o) and immobilized (•) BGP.

activity while the immobilized enzyme retained 60% activity under similar experimental conditions.

2.3.7. Effect of urea on soluble and immobilized BGP

DEAE cellulose bound BGP was more resistant to inactivation induced by 4.0 M urea compared to its soluble counterpart. Exposure of soluble enzyme with 4.0 M urea for 2 h resulted in a loss of 53% activity whereas the immobilized enzyme retained more than 70% of the initial enzyme activity under similar exposure (Fig. 6).

2.3.8. Effect of proteolysis on soluble and immobilized BGP

Figure 7 shows the stability of soluble and immobilized BGP in the presence of increasing concentrations of trypsin (0.25-2.5 mg mL^{-1}). Soluble BGP was rapidly inactivated in the presence of increasing concentrations of trypsin and retained 36% of the initial activity after 1 h incubation with 2.5 mg trypsin mL^{-1} at 37 °C while the immobilized BGP was remarkably more stable against proteolysis mediated by trypsin. However, the immobilized BGP showed over 55% of the original enzyme activity under similar treatment.

2.3.9. Effect of detergents on soluble and immobilized BGP

Here, three different detergents; Surf Excel, Rin Powder and Triton X-100, were selected for the comparative stability of soluble and immobilized BGP. Surf Excel and Rin Powder are commonly used detergents in every household and laundry. Soluble BGP was more sensitive to Surf Excel exposure and lost nearly 67% enzyme activity after 1 h incubation with 0.5% (w/v) detergent. Moreover, the immobilized BGP was markedly more resistant to inactivation induced by Surf Excel and retained 70% of the initial activity (Table 4).

Table 4 further shows the effect of increasing concentrations (0.1-1.0%, w/v) of Rin Powder on the activity of soluble and immobilized BGP. The soluble enzyme

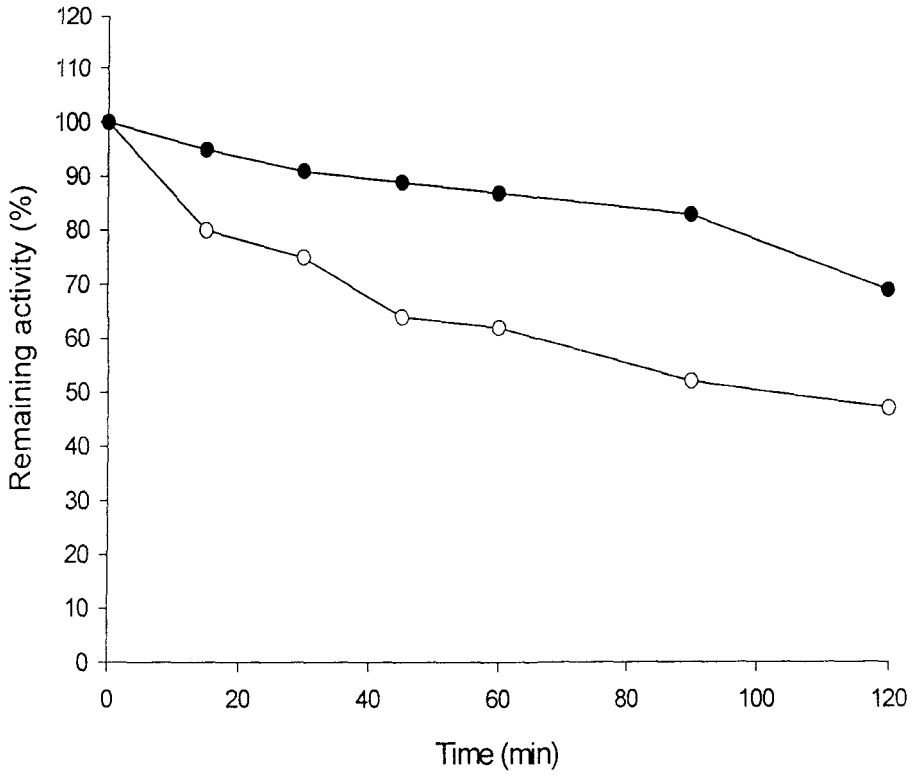

Figure 6: Effect of 4.0 M urea on soluble and immobilized BGP

Soluble and immobilized BGP (1.25 U) were incubated with 4.0 M urea in 50 mM sodium acetate buffer, pH 5.6. Enzyme activity was determined at different time intervals under conditions mentioned in the text. For calculating the remaining percent activity, urea untreated samples were considered as control (100%). Symbols indicate, the soluble (o) and immobilized (•) BGP.

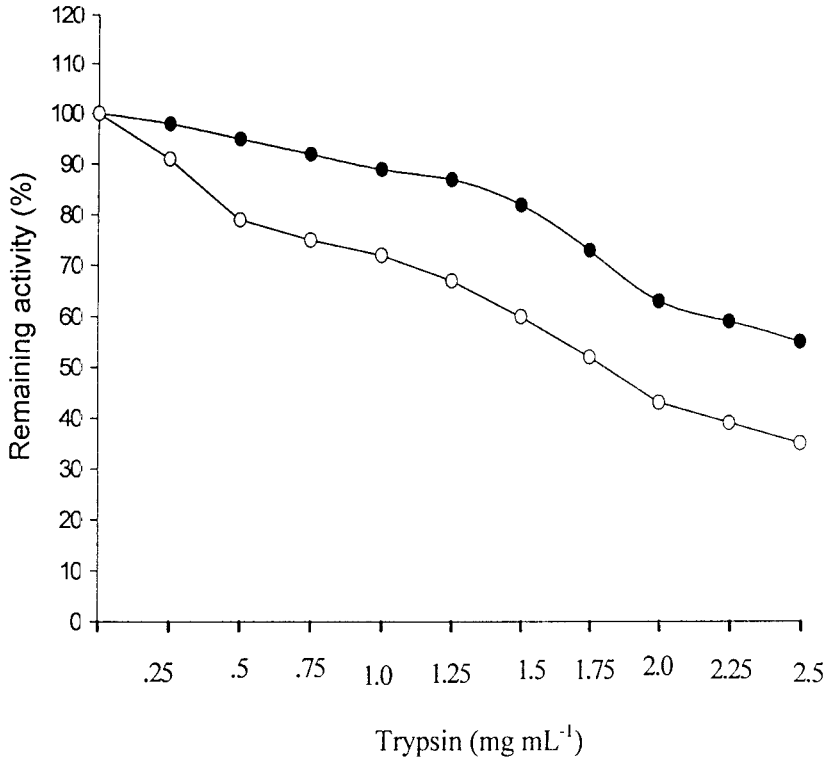

Figure 7: Effect of trypsin on soluble and immobilized BGP

Soluble and immobilized BGP (1.25 U) were independently incubated with increasing concentrations of trypsin (0.25-2.5 mg) in a total volume of 1.0 mL of 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Activity of enzyme was assayed according to the procedure described in text. Symbols indicate, the soluble (o) and immobilized (•) BGP.

Table 4: Effect of detergents on the activity of soluble and immobilized BGP

Detergent concentration (%, w/v)	Remaining activity (%)			
	Surf Excel		Rin Powder	
	SBGP	IBGP	SBGP	IBGP
0.1	105	111	99	147
0.2	94	103	98	142
0.3	69	81	93	137
0.4	57	77	83	127
0.5	33	70	58	85
0.6	22	58	25	60
0.7	21	52	20	49
0.8	14	46	12	32
0.9	11	26	8	25
1.0	7	22	6	21

Soluble and immobilized BGP (1.25 U) were incubated with varying concentrations of Surf Excel and Rin Powder (0.1-1.0%, w/v) in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated detergent concentrations and other assay conditions were same as mentioned in the text. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation < 5%.

retained 58% of the initial activity after 1 h exposure to 0.5% Rin Powder. However, the immobilized BGP exhibited 85% of the original activity under similar exposure.

Moreover, the immobilized BGP preparation was more resistant to denaturation induced by Triton X-100, this preparation retained 60% of the initial activity even in the presence of 5.0% (v/v) Triton X-100 whereas soluble BGP retained only 38% of the original activity under identical treatment (Fig. 8).

2.3.10. Effect of water-miscible organic solvents on soluble and immobilized BGP

Table 5 demonstrates the effect of water miscible-organic solvents on the activity of soluble and immobilized BGP. The exposure of soluble enzyme with varying concentrations of DMSO (10-60%, v/v) resulted in the loss of greater fraction of enzyme activity while the immobilized enzyme was quite resistant to inactivation induced by DMSO. Exposure of immobilized enzyme preparation with 50% (v/v) DMSO for 1 h retained 63% of the original activity, although the soluble enzyme lost nearly 59% of its original activity under similar treatment (Table 5). The incubation of soluble and immobilized BGP with increasing concentrations of dioxane resulted in a continuous loss of enzyme activity. However, the immobilized enzyme was more resistant to inactivation mediated by dioxane. The treatment of soluble BGP with 60% (v/v) dioxane for 1 h resulted in a loss of 84% of the initial activity while the DEAE cellulose bound BGP exhibited stabilization against similar treatment and retained 35% of its original activity (Table 5). Table 5 summarizes the treatment of soluble and immobilized BGP with increasing concentrations of *n*-propanol. The incubation of soluble enzyme with 60% (v/v) concentration of *n*-propanol resulted in decreasing more than 50% activity while immobilized enzyme showed retention of 70% of the initial activity.

2.4. DISCUSSION

Several methods have been used for the immobilization/stabilization of enzymes but very few can meet the requirement of enzyme immobilization directly from the crude

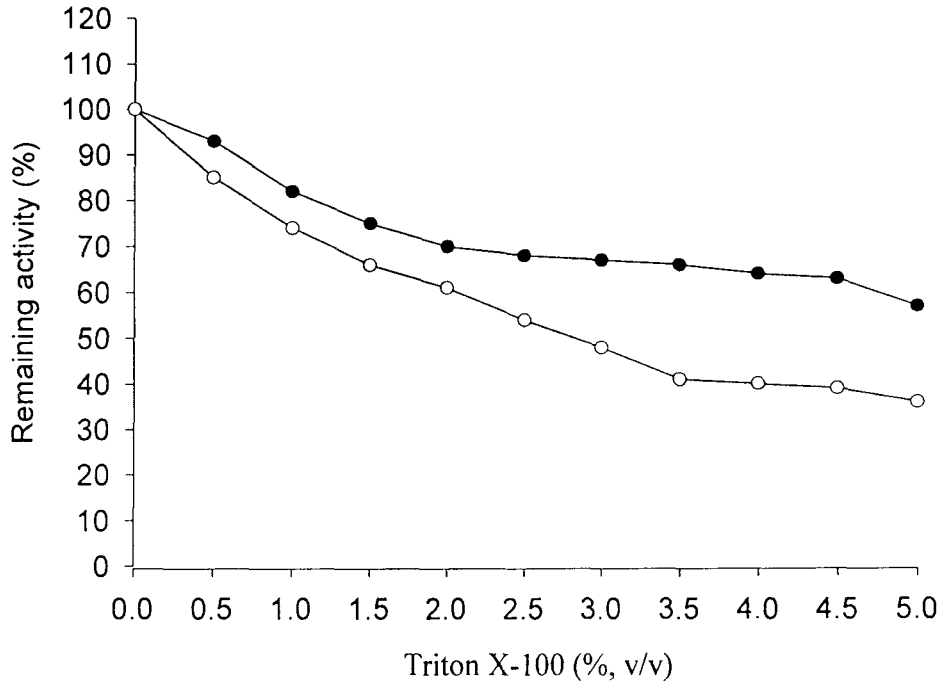

Figure 8: Effect of Triton X-100 on soluble and immobilized BGP

Soluble and immobilized BGP (1.25 U) were incubated with (0.5-5.0%, v/v) Triton X-100 prepared in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Enzyme activity was determined after incubation period as described in the text. The activity of soluble and immobilized BGP in assay buffer without any detergent was taken as control (100%) for the calculation of remaining percent activity. Symbols indicate, the soluble (o) and immobilized (•) BGP.

Table 5: Effect of water-miscible organic solvents on soluble and immobilized BGP

Organic solvent (%, v/v)	Remaining activity (%)					
	Dioxane		DMSO		*n*-Propanol	
	SBGP	IBGP	SBGP	IBGP	SBGP	IBGP
10	80	92	86	100	88	100
20	53	71	80	98	77	95
30	32	48	75	95	74	92
40	21	40	62	74	68	88
50	17	38	41	63	62	77
60	16	35	35	60	48	70

Soluble and immobilized BGP (1.25 U) were incubated with increasing concentrations of dioxane/DMSO/ *n*-propanol (0-60 %, v/v) in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated organic solvent concentrations and other assay conditions were same as mentioned in the text. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation < 5%.

homogenate (Khan et al., 2005). In this work, an effort has been made to immobilize the peroxidases directly from the ammonium sulphate fractionated proteins of bitter gourd on DEAE cellulose. It is now well documented that anion exchangers can be used for the purification of large number of proteins (Sakharov et al., 2000). BGP was immobilized in very high yield on DEAE cellulose and it could bind 590 U g^{-1} of the ion exchanger. The preparation thus obtained was highly active and exhibited very high effectiveness factor 'η' as 0.95 (Table 3). Effectiveness factor of an immobilized enzyme is a measure of internal diffusion and reflects the efficiency of the immobilization procedure (Muller and Zwing, 1982). In this procedure, the yield of immobilization was quite superior over other methods used for the immobilization of peroxidases (Lobarzewsky and Ginalska, 1995; Shaffiqu et al., 2002; Levy et al., 2003). DEAE cellulose adsorbed BGP was tightly retained as it had no significant detachment even in the presence of 0.5 M NaCl (Fig. 1). These observations suggested that the binding of BGP with DEAE cellulose was quite strong and this preparation can be easily exploited for its use in the treatment of wastewater containing aromatic pollutants.

BGP bound to DEAE cellulose support exhibited very high stabilization against denaturation mediated by pH, heat and urea (Figs. 3-6). Several earlier investigators have also reported the use of DEAE cellulose support for high yield and stable immobilization of enzymes and proteins (Strauss et al., 2000; Reddy et al., 2004). The immobilized BGP preparation exhibited broadening in pH-activity profile (Fig. 3). We have earlier shown that the Con A-Sephadex bound BGP had similar type of broadening in pH-activity profiles (Akhtar et al., 2005c). DEAE cellulose bound BGP was remarkably stable against proteolysis mediated by trypsin (Fig. 7). It has earlier been reported that Con A-Sephadex bound BGP was quite resistant to proteolysis induced by trypsin (Akhtar et al., 2005c).

Wastewater coming out from various elimination sites contains several types of denaturants including detergents, which can strongly denature the enzymes used for the treatment of polluted wastewater. In order to use such enzymes for the removal of aromatic pollutants from wastewater it becomes necessary to monitor the stability of enzymes in presence of denaturants. The effect of detergents on the activity of immobilized BGP must be investigated prior to its application in the treatment of

wastewater contaminated with hazardous organic compounds. Our observations suggested that DEAE cellulose bound BGP preparation was significantly more stable against the exposure caused by very high concentrations of several detergents (Table 4; Fig. 8). DEAE cellulose bound enzyme could work more efficiently on industrial effluents containing compounds like soaps and detergents. DEAE cellulose bound BGP was quite resistant against denaturation induced by detergents such as Triton X-100 (Fig. 8), Rin Powder and Surf Excel (Table 4). Lower concentrations of detergents enhanced the activity of immobilized BGP. These observations indicated that the presence of lower concentrations of detergents was not harmful to the enzyme's native conformation. The enhancement of enzyme activity by lower concentrations of detergents and stabilization of bioaffinity bound BGP against high concentrations of such type of detergents had already been reported by some earlier workers (Akhtar et al., 2005c).

Organic solvents are also very common pollutants together with aromatic compounds and their presence can affect the structure of enzymes. Enzymes employed for the treatment of wastewater containing pollutants would be affected by the presence of such solvents. Due to presence of organic solvents in wastewater it necessitates the investigation of the stability of enzymes against inactivation induced by the exposure of such organic solvents. The DEAE cellulose bound BGP was markedly more stable when it was exposed to dioxane, DMSO and *n*-propanol (Table 5). There have been reports that immobilization of enzymes by multipoint attachment protects them from denaturation induced by organic solvents in cosolvent mixtures (Mozhaev et al., 1990; Fernandez-Lafuente et al., 1995). Some workers have described that potato polyphenol oxidase adsorbed on chitin behaved differently compared to soluble enzyme in aqueous-organic cosolvent mixtures. Moreover, they have evaluated that enzymes; polyphenol oxidase, peroxidase, trypsin and acid phosphatase showed stimulation of enzyme activity within a specific concentration range of water-miscible organic solvent present in the medium (Batra and Gupta, 1994a). Enzyme immobilized by adsorption on Eudragit S-100, chitin and chitosan exhibited enhanced activity in organic co-solvent mixtures when the concentration of the organic solvent was around 10-20% (v/v) (Batra and Gupta, 1994b; Batra et al., 1997). More recently, in our laboratory it has been shown that enzymes

immobilized on protein supports were also quite resistant to denaturation induced by various water-miscible organic solvents (Jan et al., 2001; Jan and Husain, 2004).

DEAE cellulose bound BGP preparation has pronounced stability against pH, heat, urea, proteolysis, detergents and water-miscible organic solvents. Several earlier investigators have described that the immobilization of enzymes on DEAE cellulose support resulted in the stabilization of enzymes against various forms of denaturation (Sakharov et al., 2000; Musthapa et al., 2004; Reddy et al., 2004). Protease resistance is an additional attribute to the adsorption of BGP on an anion exchanger. It is expected that DEAE cellulose bound BGP preparation has a great future in the treatment of organic pollutants present in industrial effluents. DEAE cellulose adsorbed enzyme has only non-covalent forces between the support and enzyme molecules and sometimes it led to the desorption of enzyme from the support. Adsorbed enzyme could be cross-linked by using bifunctional or multifunctional reagents in order to prevent the dissociation/desorption of enzyme from the ion exchanger (Husain and Saleemuddin, 1989; Musthapa et al., 2004).

Adsorption procedures are significantly useful in the immobilization of enzymes directly from the crude homogenate and thus avoiding the high cost of enzyme purification. The treatment of organic pollutants present in industrial effluents by peroxidases has attracted considerable interest since past two decades. However, practical applications of large-scale enzymatic removal have always been limited due to high cost and lower operational stability of peroxidases. Moreover, this procedure emphasized the immobilization of BGP directly from the crude homogenate or ammonium sulphate precipitated proteins. It has further reduced the cost of immobilized enzyme preparation. BGP adsorbed on DEAE cellulose support showed very high yield of immobilization and markedly high stabilization against several types of denaturants. In near future, the enzyme reactors containing such inexpensive immobilized enzyme preparations could be exploited for the treatment of wastewater containing toxic and hazardous compounds.

CHAPTER-III

Bioaffinity Based an Inexpensive and High Yield Procedure for the Immobilization of Turnip (*Brassica rapa*) Peroxidase

3.1. INTRODUCTION

Numerous efforts have been made to develop the procedures for the immobilization of peroxidase from various sources but most of the immobilized enzyme preparations either use commercially available enzyme or expensive supports which increased the cost of the processes (Husain and Jan, 2000). However, such immobilized enzyme preparations could not be exploited for the treatment of large volume of effluents coming out of the industrial sites. Among the techniques used for the immobilization of enzymes, bioaffinity supports have attracted the attention of the enzymologists due to several merits over the other known classical methods. Researchers have shown remarkable interest in the immobilization of enzymes on bioaffinity-supports due to ease of immobilization, lack of chemical modification and usually accompanying an enhancement in stability (Saleemuddin and Husain, 1991; Saleemuddin, 1999). Besides the mentioned advantages offered by the bioaffinity-based procedures, there is an additional benefit, such as proper orientation of enzyme on the support (Turkova, 1999; Mislovicova et al., 2000; Khan et al., 2005). These supports have been used for the high yield and stable immobilization of glycoenzymes/enzymes. A large number of bioaffinity-based procedures have already been developed for the immobilization of enzymes directly from the crude homogenate or partially purified enzyme preparation (Saleemuddin and Husain, 1991; Saleemuddin, 1999; Akhtar et al., 2005c; Khan et al., 2005; Matto and Husain, 2006).

In this work an effort has been made to select an inexpensive and easily available source of peroxidase, turnip. The purpose of this study was to find a cheaper and easily available alternative for the commercially available enzymes and its immobilization and utilization at large scale. Con A-cellulose immobilized TP preparation was compared with its soluble counterpart for its stability against various physical and chemical denaturing agents.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Methyl α-D-mannopyranoside was the products of Sigma Chem. Co. (St. Louis, MO), USA. Jack bean meal was procured from the Loba Chem. Co., India. *o*-dianisidine-HCl was obtained from IGIB, New Delhi, India. Cetyltrimethylammonium bromide, dioxane, dimethyl formamide and *n*-propanol were obtained from the SRL Chemicals, Mumbai, India. Cellulose powder (0.02-0.15mm) was obtained from Centron Research Labs, Mumbai, India. Surf Excel was the product of Hindustan Lever Ltd., Mumbai, India. Turnip roots were purchased from the local vegetable market. Other chemicals and reagents employed were of analytical grade and used without any further purification.

3.2.2. Ammonium sulphate fractionation of turnip proteins

Turnip root (200 g) was homogenized in 200 mL of 100 mM sodium acetate buffer, pH 5.5. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000 x *g* on a Remi Cooling Centrifuge C-24. The clear solution thus obtained was subjected to salt fractionation by adding 20-80% (w/v) $(NH_4)_2SO_4$. The mixture was stirred overnight at 4 °C to obtain the maximum precipitate. The precipitate was collected by centrifugation at 10,000 x *g* on a Remi Cooling Centrifuge C-24. The obtained precipitate was re-dissolved in 100 mM sodium acetate buffer, pH 5.5 and dialyzed against the assay buffer (Matto and Husain, 2006).

3.2.3. Preparation of bioaffinity support

Cellulose (5.0 g) was incubated and stirred with 100 mL of clear solution of jack bean extract prepared in 100 mM sodium phosphate buffer, pH 6.2, overnight at 4 °C. Unbound proteins were removed by extensive washing with the assay buffer (Akhtar et

al., 2005c). The specific binding of Con A with cellulose was confirmed by eluting the bound lectin using 1.0 M methyl α-D-mannopyranoside.

3.2.4. Immobilization of TP on Con A-cellulose support

TP (7240 U) was added to 5.0 g of Con A-cellulose support and stirred in 100 mM sodium phosphate buffer, pH 6.2, at 4 °C overnight. Unbound TP was removed by extensive washing with the assay buffer (Akhtar et al., 2005c).

3.2.5. Effect of ion concentrations on Con A-cellulose TP

The immobilized TP (1.15 U) was incubated with increasing concentrations of NaCl (0.1-1.0 M) in 100 mM sodium acetate buffer, pH 5.5 for 1 h at 37 °C. Peroxidase activity was determined at all the indicated salt concentrations after the incubation.

3.2.6. Effect of pH on soluble and immobilized TP

The activity of soluble and immobilized TP (1.15 U) was measured in the buffers of various pH values (3.0–10.0). The molarity of each buffer was 100 mM.

3.2.7. Effect of temperature on soluble and immobilized TP

The activity of soluble and immobilized TP (1.15 U) was measured at various temperatures (20-80 °C) under standard assay conditions. The activity obtained at 30 °C was taken as control (100%) for the calculation of remaining percent activity.

Soluble and immobilized TP (1.15 U) were incubated at 60 °C in 100 mM sodium acetate buffer, pH 5.5. Aliquots of each preparation were removed at indicated time interval and activity was measured. The activity obtained without incubation at 60 °C was taken as control (100%) for the calculation of remaining percent activity.

3.2.8. Effect of detergents on soluble and immobilized TP

Surf Excel (0.1-1.0%, w/v), CTAB (0.2-2.0%, w/v), Triton X-100 and Tween 20 (0.2-1.0%, v/v) were used as final assay concentration to observe the effect of detergents on the activity of TP. Soluble and immobilized TP (1.15 U) were incubated with detergents in 100 mM sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was determined at all the indicated detergent concentrations. The activity without exposure to detergent was taken as control (100%) for the calculation of remaining percent activity.

3.2.9. Effect of water-miscible organic solvents on soluble and immobilized TP

Soluble and immobilized TP (1.15 U) were incubated with 10-60% (v/v) of water-miscible organic solvents; DMF/dioxane/*n*-propanol prepared in 100 mM sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was determined at all the indicated organic solvent concentrations after the incubation (Jan et al., 2001). Other assay conditions were the same as described in the text. The activity of enzyme without exposure to organic solvent was considered as control (100%) for the calculation of remaining percent activity.

3.2.10. Measurement of peroxidase activity

Peroxidase activity was estimated according to the procedure described in chapter-II.

3.2.11. Determination of protein concentration

Protein concentration was determined according to the procedure described in chapter-II.

3.3. RESULTS

3.3.1. Preparation of bioaffinity support and immobilization of TP

Cellulose adsorbed nearly 30 mg protein g^{-1} cellulose powder from jack bean extract. Con A-cellulose matrix was selected as a bioaffinity media for the direct immobilization of glycoenzymes from ammonium sulphate fractionated turnip proteins. It has already been described that some of the isoenzymes of turnip peroxidase are glycosylated (Duarte-Vazquez et al., 2003). In view of the glycoproteinic nature of turnip peroxidases, these enzymes could be directly immobilized on Con A-cellulose support from ammonium sulphate fractionated proteins or from the crude homogenate of turnip. Unbound proteins were removed by extensive washing with assay buffer. Con A-cellulose adsorbed peroxidase activity 672 U g^{-1} of the matrix (Table 6).

The stability of soluble and Con A-cellulose bound TP preparations was monitored against various physical and chemical denaturing agents because these parameters can influence the activity of enzymes used for the treatment of organic pollutants present in the wastewater.

3.3.2. Effect of ion concentrations on the Con A-cellulose bound TP

The exposure of immobilized enzyme with increasing concentrations of NaCl (0.1-1.0 M) for 1 h exhibited retention of very high enzyme activity even in the presence of 1.0 M NaCl. The incubation of immobilized TP upto 0.8 M NaCl for 1 h had no detachment of enzyme activity (Fig. 9).

3.3.3. pH-activity profiles of soluble and immobilized TP

Figure 10 shows the pH-activity profiles of soluble and Con A-cellulose bound TP. Both soluble and immobilized TP preparations showed same pH-optima at pH 5.0. However, the immobilized TP preparation exhibited higher fractions of catalytic activity at alkaline side of pH-optima.

Table 6: Immobilization of TP on Con A-cellulose support

Amount of enzyme activity loaded (X) (U)	Amount of enzyme activity in washes (Y) (U)	Activity bound g^{-1} Con A-cellulose support (U)			Activity yield (%) (B/Ax100)
		Theoretical (X-Y=A) (A)	Actual (B)	Effectiveness factor (η) (B/A)	
1448	608	840	672	0.8	80

Peroxidase activity was assayed according to the procedure described in the text. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation < 5%.

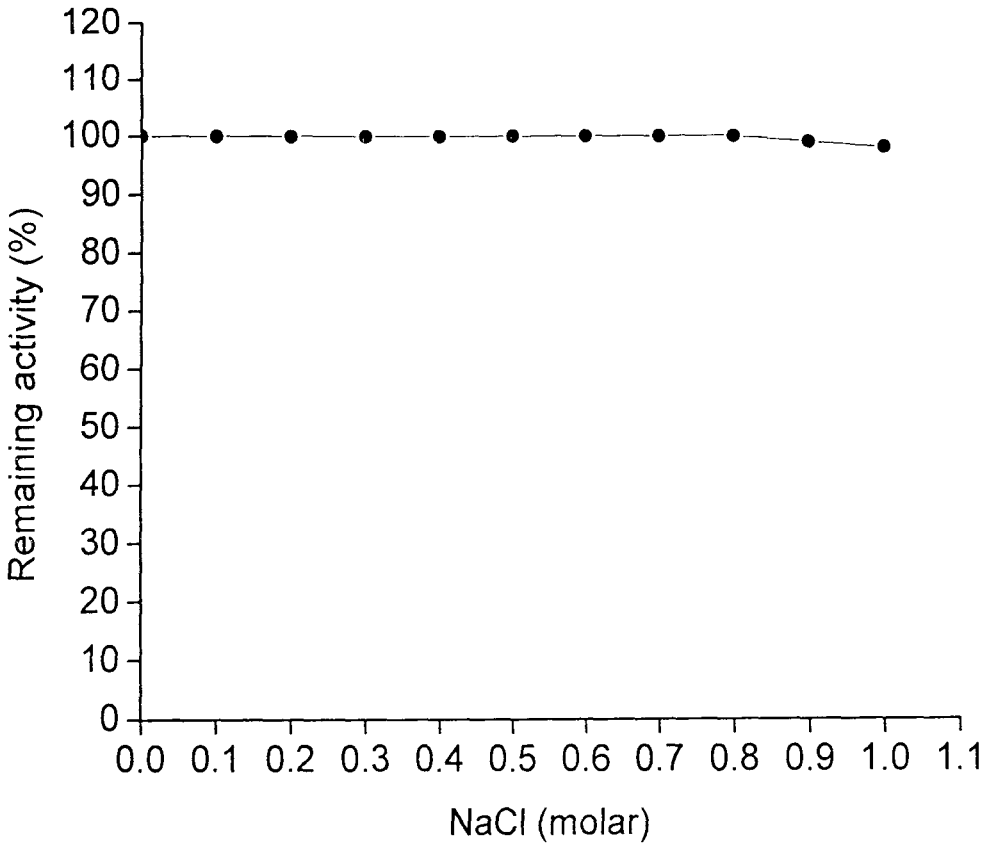

Figure 9: Effect of NaCl concentrations on Con A-cellulose bound TP

Con A-cellulose bound TP (1.15 U) was incubated with increasing concentrations of NaCl (0.1-1.0 M) in 100 mM sodium acetate buffer, pH 5.5 for 1 h at 37 °C. After incubation period each treated enzyme preparation was centrifuged to monitor the remaining adsorbed enzyme activity. Symbol indicates immobilized (•) TP.

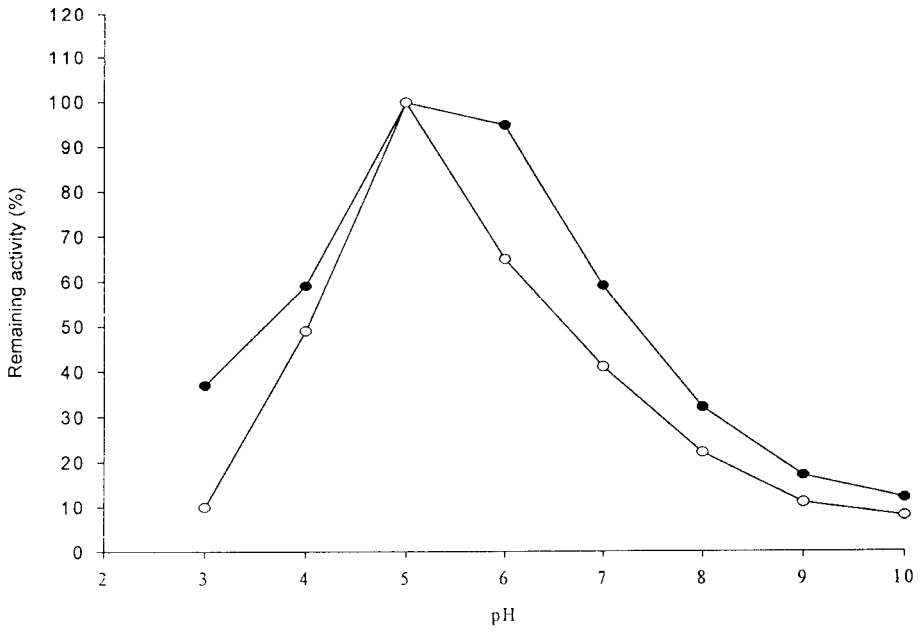

Figure 10: pH-activity profiles of soluble and immobilized TP

The appropriate amount of soluble and immobilized TP was taken for the preparation of pH-activity profiles. The reaction was carried out at 37 °C for 15 min in buffers of pH ranges from 3.0-10.0. The buffers used were glycine-HCl for pH 3.0, sodium acetate for pH 4.0, 5.0, sodium phosphate for pH 6.0-8.0 and Tris-HCl for pH 9.0 and 10.0. The molarity of each buffer was 100 mM. Activity expressed at pH 5.0 was considered as control (100%) for the calculation of remaining percent activity. Symbols indicate, the soluble (o) and immobilized (•) TP.

3.3.4. Effect of temperature on soluble and immobilized TP

Bioaffinity bound TP preparation exhibited a marginal broadening in temperature-activity profile. Soluble and Con A-cellulose bound TP preparations showed same temperature-optima at 30 °C. Con A-cellulose adsorbed TP retained greater fractions of catalytic activity at higher temperatures as compared to the free enzyme (Fig. 11). Soluble TP retained a marginal activity of 22% at 70 °C whereas the immobilized enzyme exhibited more than half of the maximum activity.

Figure 12 demonstrates the thermal denaturation of soluble and immobilized TP at 60 °C. Immobilized TP retained 54% of the initial enzyme activity after 2 h incubation at 60 °C while the soluble enzyme lost nearly 83% of the original activity under similar incubation conditions.

3.3.4. Effect of urea and guanidinium-HCl on soluble and immobilized TP

Con A-cellulose bound TP was more resistant to inactivation induced by 4.0 M guanidinium-HCl compared to its soluble counterpart. Immobilized enzyme preparation retained more than half of the initial enzyme activity when exposed to 4.0 M guanidinium-HCl for 1 h while the soluble TP exhibited marginally 15% of the initial activity under identical experimental conditions (Table 7).

Table 7 further demonstrates the treatment of soluble and immobilized TP with 4.0 M urea for various time periods. Immobilized TP was significantly more stable against denaturation mediated by urea and this preparation retained over 85% of the enzyme activity after 2 h exposure with 4.0 M urea. However, the soluble enzyme was more sensitive to urea treatment and lost more than 65% of the initial enzyme activity after 2 h treatment with 4.0 M urea (Table 7).

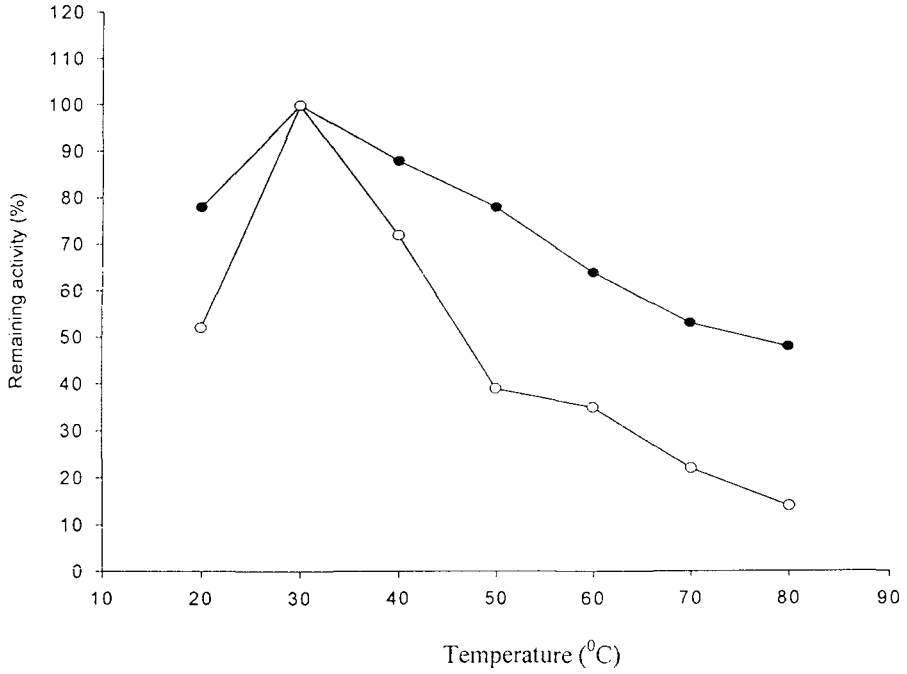

Figure 11: Temperature-activity profiles of soluble and immobilized TP

The activity of appropriate amount of soluble and immobilized TP was monitored at various indicated temperatures. Activity expressed at 30 °C was taken as control (100%) for the calculation of remaining percent activity. Symbols indicate, the soluble (o) and immobilized (•) TP.

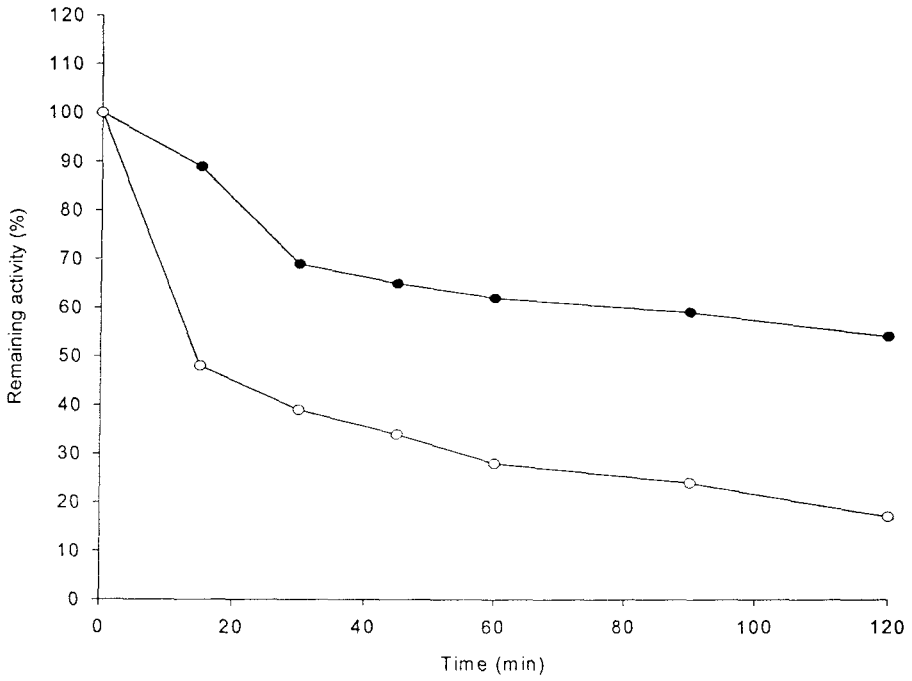

Figure 12: Thermal denaturation of soluble and immobilized TP

Soluble and immobilized TP (1.15 U) were independently incubated at 60 °C for varying time intervals in 100 mM sodium acetate buffer, pH 5.5. Aliquots of each preparation were taken out at indicated time intervals and chilled quickly in crushed ice for 5 min. After chilling, all the tubes were brought to room temperature and activity was determined as described in the text. Un-incubated samples were taken as control (100%) for the calculation of remaining percent activity. Symbols indicate, the soluble (o) and immobilized (•) TP.

Table 7: Effect of urea and guanidinium-HCl on soluble and immobilized TP

Incubation time (min)	Remaining activity (%)			
	Urea (4.0 M)		Guanidinium-HCl (4.0 M)	
	STP	ITP	STP	ITP
15	93	100	31	70
30	92	100	28	64
45	90	100	19	56
60	84	95	15	52
90	64	93	14	32
120	34	86	13	27

Soluble and immobilized TP (1.15 U) were independently incubated with 4.0 M urea/guanidinium-HCl prepared in 100 mM sodium acetate buffer, pH 5.5 at 37 °C for 2 h. Peroxidase activity was assayed at different time intervals under conditions mentioned in the text. The activity of soluble and immobilized TP in assay buffer without any denaturant was taken as control (100%) for the calculation of remaining percent activity. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation < 5%.

3.3.5. Effect of detergents on soluble and immobilized TP

Surf Excel is a very common detergent used in household and laundry. Unused detergent is normally present in the wastewater coming out of municipal waste. This wastewater is mixed with the effluents released by the industries. In order to monitor the compatibility of the immobilized enzyme in such treatments, the effect of Surf Excel on the activity of immobilized TP has been investigated. Soluble TP was more sensitive to the Surf Excel exposure and lost nearly 90% of the initial enzyme activity after 1 h exposure with 1% (w/v) detergent. However, the immobilized TP was remarkably more resistant to inactivation induced by Surf Excel and retained over 56% of the initial activity (Fig. 13). Both soluble and immobilized enzyme preparations were activated at low concentrations of Surf Excel, however, the immobilized TP achieved further activation at higher levels of detergent (Fig. 13). Lower concentrations of Triton X-100 and Tween 20 activated soluble and immobilized TP and in this case the extent of activation was significantly higher as compared to activation by Surf Excel (Table 8). In order to investigate the effect of higher concentrations of detergents on the activity of TP, soluble and immobilized TP preparations were further incubated with 0.2-2.0% (w/v) CTAB, a cationic detergent for 1 h at 37 °C. Pre-incubation of soluble and immobilized enzyme preparations with 2% (w/v) CTAB for 1 h exhibited enhanced activity of 131% and 225% of the original enzyme activity, respectively (Fig. 14).

3.3.6. Effect of water-miscible organic solvents on soluble and immobilized TP

The exposure of soluble enzyme with varying concentrations of DMF (10-60%, v/v) resulted in the loss of greater fraction of enzyme activity while the immobilized enzyme was quite resistant to inactivation induced by DMF. Con A-cellulose bound TP preparation retained nearly 70% of the original activity after 1 h exposure to 60% (v/v) DMF whereas the soluble enzyme lost 90% of the original activity under similar treatment conditions (Table 9).

The exposure of soluble and Con A-cellulose adsorbed TP with (10-60%, v/v) dioxane showed a continuous decrease in peroxidase activity. Immobilized TP retained

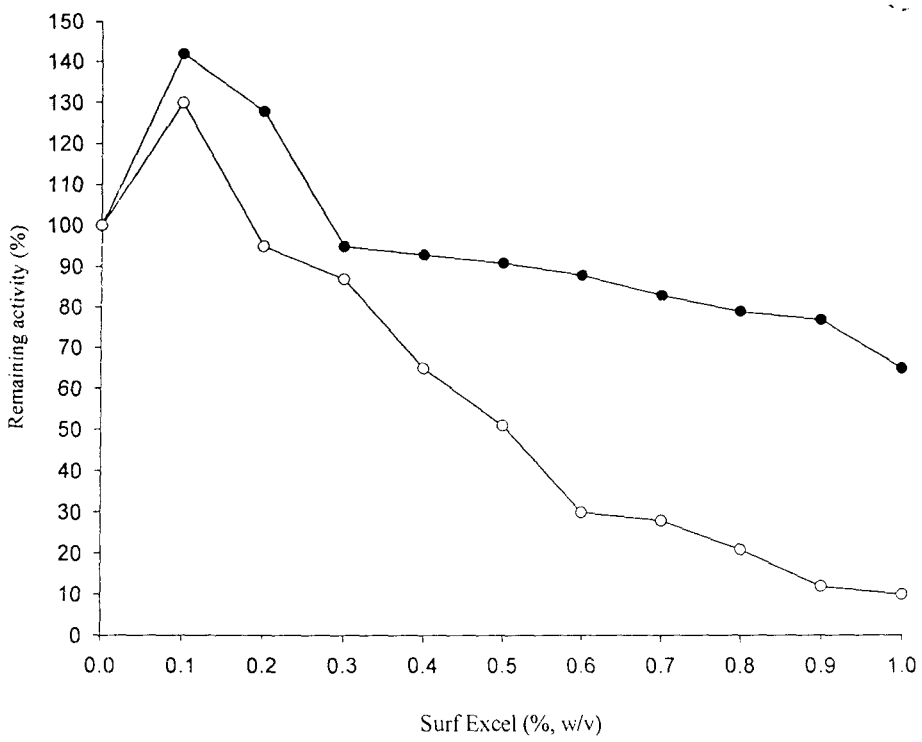

Figure 13: Effect of Surf Excel on soluble and immobilized TP

Soluble and immobilized TP (1.15 U) were incubated with varying concentrations of Surf Excel (0.1-1.0%, w/v) in 100 mM sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Enzyme activity was determined after incubation as described in the text. The activity of soluble and immobilized TP in assay buffer without any concentration of Surf Excel was taken as control (100%) for the calculation of remaining percent activity. Symbols indicate, the soluble (o) and immobilized (•) TP.

Table 8: Effect of Triton X-100 and Tween 20 on soluble and immobilized TP

Detergent (%, v/v)	Remaining activity (%)			
	Triton X-100		Tween 20	
	STP	ITP	STP	ITP
0.2	158	238	179	212
0.4	145	205	152	197
0.6	105	188	128	183
0.8	87	115	74	162
1.0	65	110	52	155

Soluble and immobilized TP (1.15 U) were independently incubated with increasing concentrations of detergent (0.2-1.0%, v/v) in 100 mM sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated detergent concentrations. The activity of soluble and immobilized TP in assay buffer without any detergent was taken as control (100%) for the calculation of remaining percent activity. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation < 5%.

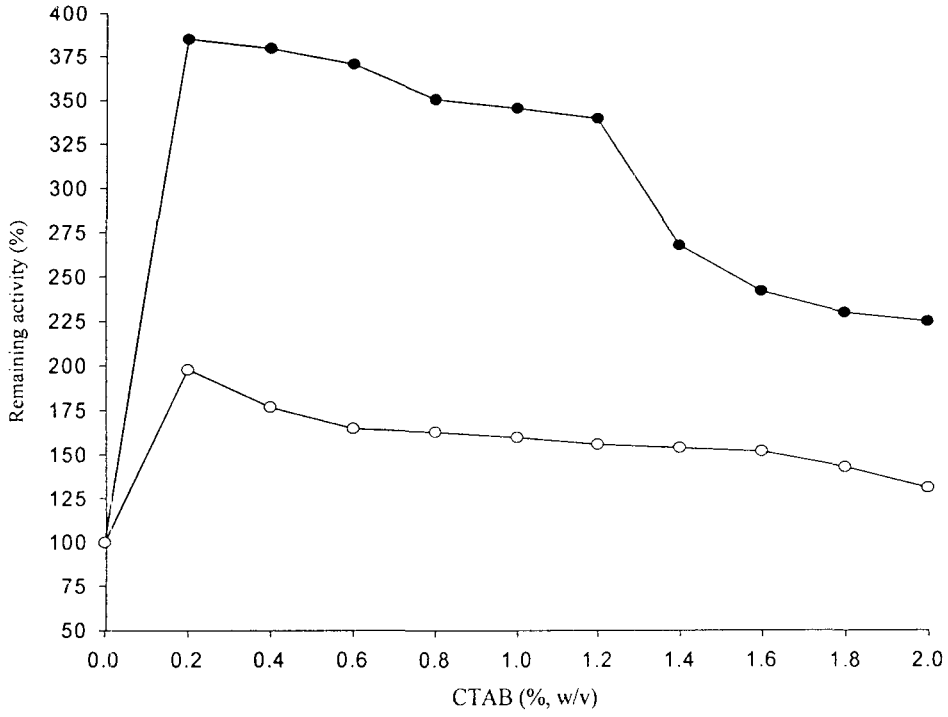

Figure 14: Effect of CTAB on soluble and immobilized TP

Soluble and immobilized TP (1.15 U) were incubated with varying concentrations of CTAB (0.2-2.0%, w/v) in 100 mM sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Enzyme activity was determined after incubation period as described in the text. The activity of soluble and immobilized TP in assay buffer without any CTAB was taken as control (100%) for the calculation of remaining percent activity. Symbols indicate, the soluble (o) and immobilized (•) TP.

Table 9: Effect of water-miscible organic solvents on soluble and immobilized TP

Organic solvent (%, v/v)	Remaining activity (%)					
	DMF		Dioxane		*n*-propanol	
	STP	ITP	STP	ITP	STP	ITP
10	87	97	76	88	76	92
20	75	80	41	58	54	62
30	56	79	26	41	20	58
40	34	78	13	38	18	57
50	20	74	11	30	16	53
60	10	69	10	23	12	51

Soluble and immobilized TP (1.15 U) were independently incubated with increasing concentrations of DMF/dioxane/*n*-propanol (10-60%, v/v) in 100 mM sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated organic solvent concentrations. The activity of soluble and immobilized TP in assay buffer without any organic solvent was taken as control (100%) for the calculation of remaining percent activity. Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation < 5%.

greater fraction of catalytic activity at various concentrations of dioxane (Table 9). The incubation of soluble and immobilized TP with increasing concentrations of *n*-propanol resulted in a continuous loss of enzyme activity. However, the immobilized enzyme preparation exhibited more resistance to inactivation induced by *n*-propanol. The treatment of soluble TP with 60% (v/v) *n*-propanol for 1 h resulted in a loss of 88% of the initial activity while the Con A-cellulose bound TP exhibited significantly higher stabilization against similar treatment and retained more than 50% of the initial activity (Table 9).

3.4. DISCUSSION

Here a simple and an elegant approach have been applied to immobilize TP directly from ammonium sulphate fractionated proteins of turnip roots on Con A-cellulose. It is now well documented that polysaccharides could be used for bioaffinity-based purification of Con A from the jack bean extract (Saleemuddin and Husain, 1991). However, such property has been exploited for the preparation of bioaffinity media for the immobilization of enzymes from crude preparations. TP was immobilized in very high yield on Con A-cellulose support and it could bind peroxidase activity 672 U g^{-1} of the adsorbent (Table 6). The immobilization yield was quite superior over other methods used for the immobilization of peroxidases (Husain et al., 1992; Akhtar et al., 2005c). Con A-cellulose bound TP exhibited very high stabilization against the inactivation induced by pH, heat, urea and guanidinium-HCl denaturation (Figs. 10-12, Table 7). Several earlier investigators have also reported about the use of Con A support for high yield and stable immobilization of glycoenzymes (Saleemuddin and Husain, 1991; Saleemuddin, 1999).

Lower concentrations of various detergents enhanced activity of soluble and immobilized TP. These experiments indicated that the presence of lower concentrations of detergents is not harmful to the enzyme function. Such enzymes can work even more efficiently on industrial effluents containing compounds like detergents. Con A-cellulose adsorbed TP was quite resistant against denaturation induced by detergents such as Triton X-100, Tween 20 (Table 8), Surf Excel and CTAB (Figs. 13 and 14). Numerous

detergents normally flow in the municipal wastewater and these could affect the activity of enzymes. Our observations suggested that Con A-cellulose bound TP preparation was remarkably more stable against the exposure caused by high concentration of several detergents.

Organic solvents are also very common pollutants along with aromatic compounds and their presence could influence the structure of enzymes. Enzymes exploited for the treatment of wastewater containing aromatic pollutants would be affected by the presence of water-miscible organic solvents. Con A-cellulose bound TP was remarkably more resistant against the inactivation mediated by DMF, dioxane and *n*-propanol (Table 9). It has already been reported that immobilization of enzymes by multipoint attachment protects them from denaturation mediated by water-miscible organic solvents (Mozhaev et al., 1990; Fernandez-Lafuente et al., 1995; Batra and Gupta, 1994a). Akhtar et al. (2005c) have demonstrated that BGP immobilized on Con A-Sephadex support behaved differently compared to soluble enzyme in aqueous-organic co-solvent mixtures. More recently, in our laboratory it has been shown that enzymes immobilized on protein supports were also quite resistant to denaturation mediated by various water-miscible organic solvents (Jan et al., 2001; Jan and Husain, 2004; Matto and Husain, 2006). Fernandes et al. (2003) improved the tolerance of HRP to organic solvents by immobilization. In a more recent study, Magri et al. (2005) have also reported that immobilized soybean seed coat peroxidase shows full activity over the organic solvent concentration range (5-70%, v/v) whereas the free enzyme was almost inactive in 50% (v/v) of the solvents. Enzymatic catalysis in organic solvents is possible if the organic solvent does not substantially disturb the active-site structure and if enough water is present (Ryu and Dordick, 1992); it seems that in the case of Con A-cellulose bound TP both conditions are met.

Con A-cellulose bound TP preparation has pronounced stability against pH, heat, urea, guanidinium-HCl, detergents and water-miscible organic solvents. Earlier reports described that the immobilization of glycoenzymes on Con A support resulted in the stabilization of enzymes against several types of denaturation (Mislovicova et al., 2000; Akhtar et al., 2005c). Therefore, it has been suggested that Con A-cellulose bound TP exhibited great potential in the treatment of organic pollutants present in industrial

effluents. Con A-cellulose adsorbed enzyme has only non-covalent forces between the support and enzyme molecules and sometimes it led to the desorption of enzyme or Con A or both from the support. Cross-linking of bioaffinity adsorbed enzyme could be done by using bifunctional or polyfunctional reagents to prevent the dissociation/desorption of enzyme or Con A-glycoenzyme complex from the cellulose support (Jan et al., 2006).

The procedure for the immobilization of proteins, developed in this study exhibited its own merits due to use of crude jack bean extract, the source of lectin and ammonium sulphate fractionated turnip proteins, source of enzyme. Moreover, this procedure emphasized the immobilization of TP directly from the crude homogenate or ammonium sulphate fractionated proteins on bioaffinity support. TP adsorbed on Con A support showed very high yield of immobilization and markedly high stabilization against several forms of denaturants. In near future, the reactors containing such types of inexpensive immobilized enzyme preparations could be exploited for the treatment of wastewater containing toxic aromatic compounds.

CHAPTER-IV

Decolorization and Degradation of Acid Dyes Mediated by Salt Fractionated Turnip (*Brassica rapa*) Peroxidases

4.1. INTRODUCTION

Pollution of communal water bodies by waste dyestuff released from textile plants and dye houses represents a major environmental concern. There is currently a considerable environmental interest for color removal from a wide range of wastewater. In case of the textile manufacturing industry, up to 15% of the dyes are lost after dyeing process that are disposed out in the effluents (McMullan et al., 2001; Robinson et al., 2001b). Most of the dyes used in textile industry are classified as cationic, anionic or nonionic type. Anionic dyes are the direct, acid and reactive dyes (O'Neill et al., 1999). Acid dyes comprises of one of the classes of water-soluble synthetic dyes with greatest variety of colors and structures. Although presently a wide range of physical and chemical methods are available to decolorize dye-contaminated effluents (Hao et al., 2000) but these methods are outdated due to some unresolved problems. However, alternative procedures based on biotechnological principles are attracting increasing interest (Robinson et al., 2001b; Kandelbauer et al., 2004).

However, the biological procedures have their own limitations such as the non-biodegradability of the xenobiotic compounds due to lack of requisite enzymes in the biological treatment plant (Robinson et al., 2001b; Keharia and Madamwar, 2003). Often the environment of the microorganisms is not optimal for rapid degradation of pollutants (McMullan et al., 2001; Robinson et al., 2001b). There is a need to find alternative procedures for their treatment that are effective in removing dyes from large volume of effluents and are low in cost (Soares et al., 2001).

Recently, enzymatic approach has attracted much interest in the removal of phenolic pollutants from aqueous solutions as an alternative strategy to the conventional chemical as well as microbial treatments that pose some serious limitations (Husain and Jan, 2000; Duran and Esposito, 2000; Torres et al., 2003). Oxidoreductive enzymes; peroxidases and polyphenol oxidases are participating in the degradation/removal of aromatic pollutants from various contaminated sites (Husain, 2006; Husain and Husain, 2007). These enzymes can act on a broad range of substrates that can also catalyze the decolorization and decontamination of organic pollutants even if they are present in a very low concentration at the contaminated site. In view of the potential of the enzymes

in treating the phenolic compounds several microbial and plant oxidoreductases have been employed for the treatment of dyes but none of them has been exploited at the large scale due to low enzymatic activity in biological materials and high cost of enzyme purification (Bhunia et al., 2001; Shaffiqu et al., 2002; Verma and Madamwar, 2002; Husain, 2006).

In this study, we have investigated the role of partially purified TP for the degradation/decolorization of acid dyes, having a wide spectrum of chemical groups, currently being used by the textile industries. Majority of the tested dyes were recalcitrant to decolorization/degradation by TP. However, the addition of 2.0 mM HOBT, a redox mediator, to the reaction mixture enhanced the rate of decolorization of these dyes several folds. The mixtures of dyes were also successfully decolorized by TP. Kinetic parameters of TP with various dyes were also determined in order to examine the affinity of the enzyme for dyes.

4.2. MATERIALS AND METHODS

4.2.1. Materials

Acid dyes were a gift from Atul Chemicals, Ltd. India. Ammonium sulphate and 1-hydroxybenzotriazole were purchased from SRL Chemicals, Mumbai, India. *o*-dianisidine-HCl was obtained from IGIB, New Delhi, India. Turnip used in the study was purchased from a local vegetable market. The chemicals and other reagents employed were of analytical grade and were used without any further purification.

4.2.2. Ammonium sulphate fractionation of turnip proteins

Turnip proteins were fractionated from the buffer extract by using ammonium sulphate as described in chapter III.

4.2.3. Treatment of dyes with increasing concentration of TP

The dyes (40-170 mg L^{-1}) were prepared in 100 mM sodium acetate buffer, pH 5.0. Each dye was incubated with increasing concentration of TP "0.117-0.352 U mL^{-1}" in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H_2O_2 for 1 h at 37 °C. HOBT (2.0 mM) was used as a redox mediator for the selected experiments. Dye decolorization by TP was monitored at their respective wavelength maxima in the presence and absence of 2.0 mM HOBT. The percent decolorization was calculated by taking untreated dye solution as control (100%).

4.2.4. Treatment of dyes with fixed concentration of TP for varying times

Each dye was incubated with "0.235 U mL^{-1}" of TP in 100 mM sodium acetate buffer, pH 5.0 at 37 °C in the presence of 0.75 mM H_2O_2 for varying time intervals; 15 min to 2 h. Decolorization was also performed in the presence of 2.0 mM HOBT under other similar experimental conditions. The decrease in absorbance was monitored at predetermined intervals at the respective λ_{max} of each dye. The percent decolorization was calculated by taking untreated dye solution as control (100%).

4.2.5. Effect of pH on the decolorization of dyes by TP

In this experiment the dyes were prepared in the buffers of different pH values (3.0-10.0). Each dye was treated with TP "0.235 U mL^{-1}" in various buffers in the presence of 0.75 mM H_2O_2 for 1 h at 37 °C. HOBT (2.0 mM) was used as a redox mediator for the dye decolorization. Dye decolorization by TP was monitored at the respective wavelength maxima of each dye. The percent decolorization was calculated by taking each untreated dye in specific buffer as control (100%).

4.2.6. Effect of temperature on the decolorization of dyes by TP

Each dye was incubated with TP "0.235 U mL^{-1}" in 100 mM sodium acetate buffer, pH 5.0 and 0.75 mM H_2O_2 at 30-80 °C for 1 h. HOBT (2.0 mM) was used as a redox mediator for the dye decolorization. Decrease in color of dyes after treatment with TP was monitored at specific wavelength maxima of each dye. The percent decolorization was calculated by incubating untreated dye at each temperature as control (100%).

4.2.7. Decolorization of mixture of dyes by TP

Dye mixtures were prepared by mixing different dyes in equal proportions in terms of absorbance. The mixtures of dyes were treated with TP "0.235 U mL^{-1}" in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H_2O_2 and 2.0 mM of HOBT for 1 h at 37 °C. Decrease in absorbance in each TP treated dye mixture was monitored at its specific wavelength maxima. The percent decolorization was calculated by taking untreated dye mixture as control (100%).

4.2.8. Determination of K_m and V_{max} of the TP with respect to tested dyes

The initial rates of enzymatic dye degradation were measured at various concentrations of the dye. In this experiment, the solutions having different dye concentrations ranging from 15-200 mg L^{-1} were treated with TP "0.235 U mL^{-1}" in 100 mM sodium acetate buffer, pH 5.0 and 0.75 mM H_2O_2 at 37 °C for 1 h. HOBT (2.0 mM) was used as a redox mediator for the dye decolorization.

4.2.9. *Allium cepa* test for TP treated dyes

The *Allium cepa* bioassay for the treated dye sample was carried out according to the method of Fiskesjo (1985). For this test small onions of equal size were taken and yellowish brown outer scales and brownish bottom plates were removed by using a sharp

knife. Care was taken to maintain the ring primordial intact. Boiling tubes filled with treated dye sample consisting of "0.235 U mL^{-1}" TP in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H_2O_2 and 2.0 mM of HOBT were used to remain in contact with onion bulbs. Aqua guard water was used as control in all experiments, and the experiments were performed in dark conditions.

One onion was placed at the top of each tube with root primordial downward touching the liquid. After a gap of 12 h the same samples were added in the tube to fill up to the top and care was taken there to prevent gap between onion bulb and sample present in the test tube.

The treatment was continued for 7 days. After completion of the time of treatment, onions were taken out and root length of each sample was measured. Inhibitions in the growth of *Allium cepa* roots were considered as an index for the degree of toxicity (Fiskesjo, 1985).

4.2.10. Determination of TOC content of control and treated textile effluents

Each dye and mixture of dyes were incubated with "0.235 U mL^{-1}" of TP in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H_2O_2 and 2.0 mM HOBT for 1 h at 37 °C. The dye solutions treated with TP was kept in boiling water bath for 15 min to stop the reaction. The treated dye and mixtures of dyes were further incubated with activated silica gel (1.0 mg mL^{-1}) for 2 h at 37 °C with constant stirring. The colored product, adsorbed on the activated silica gel, was separated from the reaction mixture by centrifugation at 3000 x *g* for 15 min. After the removal of the colored product, the TOC content of the clear supernatant was determined by using a total organic carbon analyzer (Multi N/C 2000, Analytic Jena, Germany). Each control and treated independent dye/mixture of dyes was diluted to 10-fold before measuring TOC content.

4.2.11. UV-visible spectral analysis

Procedure for the dye decolorization was followed by UV-visible spectral analysis. Spectra for the control and TP treated dye samples were taken on Cintra 10*e* UV-visible spectrophotometer.

4.2.12. Assay of TP activity

Peroxidase activity was measured according to the procedure described in chapter II.

4.2.13. Determination of protein concentration

The protein concentration was determined by the procedure described in chapter II.

4.3. RESULTS

4.3.1. Treatment of dyes by varying TP concentrations

Table 10 summarizes the decolorization of five acid textile dyes by using increasing concentrations of TP "0.117-0.352 U $mL^{-1}$of reaction volume" for 1 h at 37 °C. An increase in the enzyme activity has resulted in a continuous enhancement in the rate of dye decolorization. Acid Blue 92 was decolorized 61% with 0.352 U mL^{-1} of TP whereas Acid Red 97, Acid Yellow 42, Acid Black 1 and Acid Black 210 were recalcitrant to the TP action (Table 10).

4.3.2. Treatment of dyes for varying times with fixed concentration of TP

Acid dyes were incubated with "0.235 U mL^{-1}" of TP for increasing time period. Out of five acid dyes only Acid Blue 92 was decolorized on treatment with TP for 2 h at 37 °C. Although more color disappeared when dye was incubated for longer duration,

however the rate of decolorization was slow (Table 11). Rest of the four dyes; Acid Red 97, Acid Yellow 42, Acid Black 1 and Acid Black 210 were fully recalcitrant to decolorization by TP even after prolonged incubation under similar experimental conditions.

4.3.3. Treatment of dyes in the presence of redox-mediator

Five acid dyes used in this study were treated with TP in the presence of 2.0 mM HOBT and 0.75 mM H_2O_2 at 37 °C. Presence of HOBT drastically enhanced the rate of decolorization of recalcitrant dyes (Table 10). Acid Red 97, Acid Yellow 42, Acid Black 1 and Acid Black 210 were recalcitrant to decolorization by TP in the absence of HOBT. However, these dyes were decolorized up to 92%, 91%, 87% and 65%, respectively, by the action of TP "0.352 U mL^{-1} of reaction volume" in the presence of 2.0 mM HOBT at 37 °C for 1 h.

Role of HOBT in the decolorization of recalcitrant dyes is further noticeable in Table 11. The decolorization of dyes with 0.235 U mL^{-1} of reaction volume at 37 °C for 1 h in the presence of 2.0 mM HOBT was 86% for Acid Red 97, 91% for Acid Yellow 42, 87% for Acid Black 1 and 62% for Acid Black 210. It was evident from these results that 1 h of the reaction time was sufficient for the maximum removal of dye (Table 11). After 1 h of incubation time, a marginal amount of dye removal was noticed up to remaining 2 h of the incubation time. Acid Yellow 42 was decolorized up to 91% within 15 min of incubation time.

4.3.4. Effect of pH on the decolorization of dyes with TP

Five acid textile dyes were treated with TP in the buffers of different pH (Figure 15). Most of the dyes were maximally decolorized at pH 5.0. As pH of the decolorizing solution was increased up to pH 10.0, the rate of decolorization decreased in all the treated acid dyes. Further, at higher pH Acid Red 97 and Acid Black 210 were marginally decolorized. However, Acid Blue 92, Acid Black 1 and Acid Yellow 42 showed no decolorization of dyes in the alkaline range of pH (Fig. 15).

Table 10: Effect of TP concentration on the decolorization of acid dyes

Name of the dye	λ_{max} (nm)	Decolorization (%)					
		Enzyme (U mL^{-1})					
		0.117		0.235		0.352	
		(-)	(+)	(-)	(+)	(-)	(+)
Acid Blue 92	564	13	70	21	100	61	100
Acid Red 97	497	0	27	0	86	0	92
Acid Yellow 42	408	0	74	0	91	0	91
Acid Black 1	619	0	26	0	87	0	87
Acid Black 210	456	0	24	0	62	0	65

Each dye was treated with increasing concentration of TP (0.117-0.352 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. HOBT (2.0 mM) was used as a redox-mediator for the selected experiments. In this table (-) and (+) sign indicate the decolorization of dyes in the absence and presence of HOBT, respectively. Each value represents the mean for three independent experiments performed in duplicate with average standard deviation <5 %.

Table 11: Treatment of acid dyes with fixed concentration of TP for varying times

Name of the dye	Decolorization (%)											
	Time (min)											
	15		30		45		60		90		120	
	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
Acid Blue 92	7	68	15	76	17	98	21	100	22	100	25	100
Acid Red 97	0	72	0	80	0	84	0	86	0	91	0	92
Acid Yellow 42	0	91	0	91	0	91	0	91	0	91	0	91
Acid Black 1	0	73	0	75	0	77	0	87	0	87	0	87
Acid Black 210	0	37	0	41	0	62	0	62	0	63	0	65

Each dye was treated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for varying time periods. HOBT (2.0 mM) was used as a redox-mediator for the selected experiments. In this table (-) and (+) sign indicate the decolorization of dyes in the absence and presence of HOBT, respectively. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5 %.

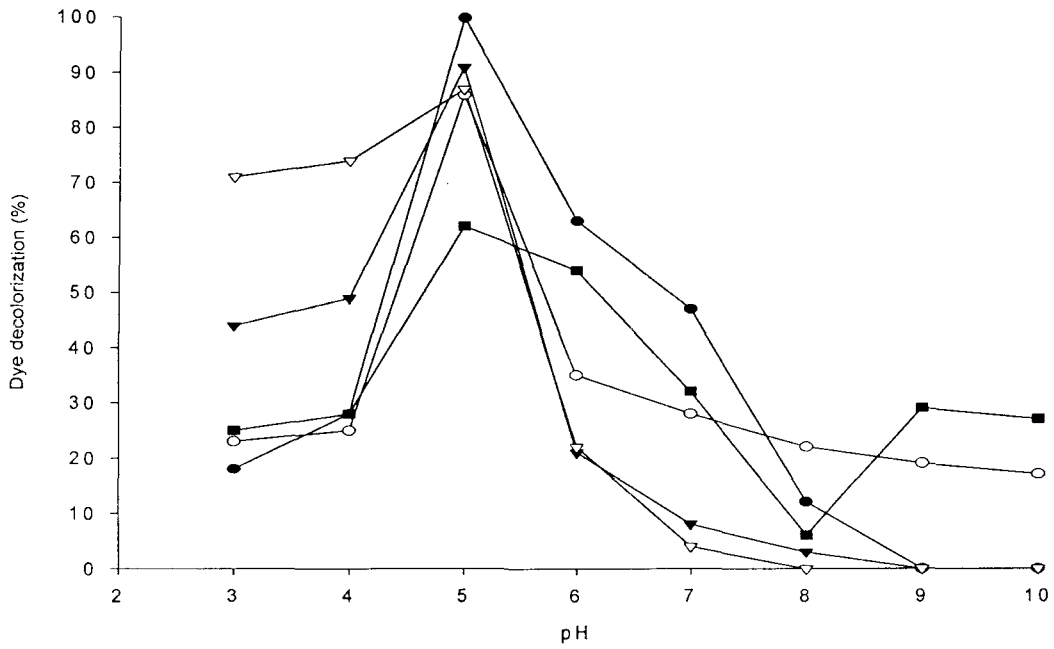

Figure 15: Effect of pH on the TP mediated decolorization of acid dyes

Each dye was treated with TP (0.235 U mL^{-1}) in buffers of different pH (3.0-10.0) at 37 °C for 1 h. HOBT (2.0 mM) was used as a redox-mediator. Symbols indicate (●) Acid Blue 92, (○) Acid Red 97, (▼) Acid Yellow 42, (▽) Acid Black 1 and (■) Acid Black 210.

4.3.5. Effect of temperature on the decolorization of dyes with TP

Figure 16 shows the effect of different temperatures (30-80 °C) on the decorization of acid dyes. The decolorization of dyes was maximum at 40 °C. Above and below this temperature rate of dcolorization was significantly decreased.

4.3.6. Treatment of mixtures of dyes with TP

To understand the decolorization of dyes present in industrial effluents, the complex mixtures of various acid dyes were prepared by mixing different dyes in equal proportions and incubated with "0.235 U mL^{-1}" of TP in the presence of 2.0 mM HOBT and 0.75 mM H_2O_2 for 1 h at 37 °C. The λ_{max} for each dye mixture was pre-determined and decolorization of mixtures was monitored after incubation period at their λ_{max}. The mixtures were decolorized to the varying extent (37-95%) in the presence of 2.0 mM HOBT whereas the complex mixtures were also recalcitrant to the action of TP in the absence of redox-mediator (Table 12).

4.3.7. Kinetics of acid dye decolorization

In order to determine the kinetic parameters of enzyme for various dyes, an experiment with different dye concentrations, ranging from 15 to 200 mg L^{-1} was performed. The plot of initial rate vs dye concentration for all five acid dyes followed the hyperbolic pattern as expected for Michaelis-Menten kinetics (data not given). Moreover, the Lineweaver-Burk plot, inverse of initial rate vs inverse of substrate (dye) concentration is also found to be linear. The values of specific dye decolorization rate ($r_{dye\ max}$) estimated from the experimental data was 7.5 mg $L^{-1}h^{-1}$ for Acid Blue 92, 79 mg $L^{-1}h^{-1}$ for Acid Red 97, 6.5 mg $L^{-1}h^{-1}$ for Acid yellow 42, 4.2 mg $L^{-1}h^{-1}$ for Acid Black 1 and 20.5 mg $L^{-1}h^{-1}$ for Acid Black 210. The value of apparent Michaelis constant (K_m) was 89 mg L^{-1} for Acid Blue 92, 97.5 mg L^{-1} for Acid Red 97, 6.2 mg L^{-1} for Acid

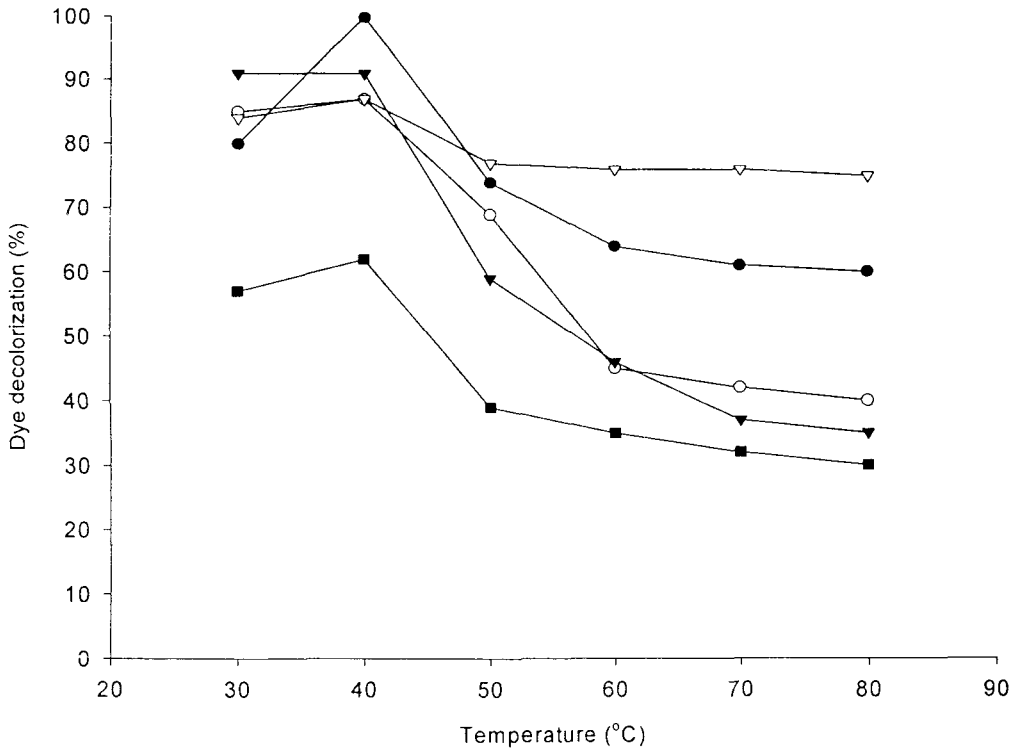

Figure 16: Effect of temperature on the TP mediated decolorization of acid dyes

Each dye was treated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 30-80 °C for 1 h. HOBT (2.0 mM) was used as a redox-mediator. Symbols indicate (●) Acid Blue 92, (○) Acid Red 97, (▼) Acid Yellow 42, (▽) Acid Black 1 and (■) Acid Black 210.

Table 12: Decolorization of polluted water-containing mixture of dyes

Mixture of acid dyes	λ_{max} (nm)	Decolorization (%)	
		(+)HOBT	(-)HOBT
Acid Red 97 + Acid Yellow 42 + Acid Black 1+ Acid Blue 92	541	85	6
Acid Yellow 42 + Acid Black 1+ Acid Black 210+Acid Blue 92	582	77	0
Acid Red 97 + Acid Black 210+ Acid Black 1+ Acid Blue 92	580	95	4
Acid Red 97 + Acid Yellow 42 + Acid Blue 92 + Acid Black 210	531	41	0
Acid Red 97 + Acid Yellow 42 + Acid Black 1+ Acid Black 210	439	37	0
Acid Red 97 + Acid Yellow 42 + Acid Black 1+ Acid Black 210+Acid Blue 92	536	57	0

The mixtures of dyes were treated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. HOBT (2.0 mM) was used as a redox-mediator for the selected experiments. In this table (-) and (+) sign indicate the decolorization of dyes in the absence and presence of HOBT, respectively. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5 %.

Table 13: Kinetic constants of TP for decolorization of dyes

Name of the dye	$r_{dye\ max}$ (mg L^{-1} h^{-1})	Km (mg L^{-1})
Acid Blue 92	7.5	89
Acid Red 97	79	97.5
Acid Yellow 42	6.5	6.2
Acid Black 1	4.2	35.8
Acid Black 210	20.5	16

Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5 %.

Yellow 42, 35.8 mg L^{-1} for Acid Black 1 and 16 mg L^{-1} for Acid Black 210 (Table 13). The Km value was lowest for the Acid Yellow 42, it showed the highest affinity of the enzyme for this dye. It was also evident from Table 13 that in the presence of HOBT the Acid Yellow 42 was decolorized 91% in 15 min. However, other dyes took more time to achieve the same decolorization.

4.3.8. Determination of phytotoxicity of decolorized product

In order to examine the toxicity of TP decolorized product of acid dyes, we perform phytotoxicity experiment by using *Allium cepa* test with all the used dyes and their decolorized product. Table 14 shows the growth of *Allium cepa* in terms of length in cm and percent inhibition brought about by dye solutions (untreated and treated). When *Allium cepa* was incubated with dye solutions for 7 days the maximum growth inhibition was recorded to be 94.54% for Acid Red 97 (untreated), the average root length was recorded to be 0.30 cm compared with 5.50 cm in control. The minimum inhibition in root length was 65.45% for TP treated Acid Yellow 42 product as compared to control.

Table 14 also shows the effect of Acid Black 1 on the *Allium cepa* root inhibition, which further demonstrates the formation of nontoxic dye degraded products. However, the degradation of Acid Blue 92 and Acid Black 210 brought about a growth inhibition of 84.54% and 85.09%, respectively, which was high in comparison to untreated dye solutions.

4.3.9. TOC content of TP treated dyes

The TOC content of individual dye and mixtures of dyes before and after treatment with TP is given in Tables 15 and 16, respectively. As compared to control the level of TOC was decreased in all the TP treated solutions.

Table 14: Length of *Allium cepa* roots and percent inhibition by TP decolorized acid dyes

Name of the dye	Test solution	Root length (cm)	Inhibition (%)
	Control	5.50	-
Acid Blue 92	Untreated	1.65	70.00
	Treated	0.85	84.54
Acid Red 97	Untreated	0.30	94.54
	Treated	1.30	76.36
Acid Yellow 42	Untreated	1.30	76.36
	Treated	1.90	65.45
Acid Black 1	Untreated	0.80	85.45
	Treated	0.90	83.36
Acid Black 210	Untreated	1.10	80.00
	Treated	0.82	85.09

Each dye was treated as described in the text. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5 %.

Table 15: TOC content of acid dyes after treatment with TP

Name of the dye	TOC content (ppm)	
	Control	Treated
Acid Blue 92	236	58
Acid Red 97	240	49
Acid Yellow 42	248	37
Acid Black 1	269	41
Acid Black 210	246	85

Each dye was treated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. HOBT (2.0 mM) was used as a redox-mediator. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5 %.

Table 16: TOC content of mixture of acid dyes after treatment with TP

Mixture of acid dyes	TOC content (ppm)	
	Control	Treated
Acid Red 97 + Acid Yellow 42 + Acid Black 1+ Acid Blue 92	225	78
Acid Yellow 42 + Acid Black 1+ Acid Black 210+Acid Blue 92	205	92
Acid Red 97 + Acid Black 210+ Acid Black 1+ Acid Blue 92	232	69
Acid Red 97 + Acid Yellow 42 + Acid Blue 92 + Acid Black 210	249	89
Acid Red 97 + Acid Yellow 42 + Acid Black 1+ Acid Black 210	216	73
Acid Red 97 + Acid Yellow 42 + Acid Black 1+ Acid Black 210+Acid Blue 92	209	85

Each mixture was treated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h. HOBT (2.0 mM) was used as a redox-mediator. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5 %.

4.3.10. UV-visible spectral analysis

Figures 17 and 18 demonstrate the decolorization and degradation of Acid Blue 92 and a mixture comprised of Acid Red 97, Acid Yellow 42, Acid Blue 92, and Acid Black 1, respectively by TP in the presence of 2.0 mM HOBT. These spectra evidently demonstrate the rapid disappearance of absorption peaks in the visible region in the presence of HOBT.

4.4. DISCUSSION

TP catalyzed polymerization and precipitation of various phenolic compounds is well documented in the literature (Duarte-Vazquez et al., 2002; Singh and Singh, 2002). However, the decolorization and degradation of dyes by TP has not yet been reported. Therefore, for the first time we have investigated the role of TP in the decolorization of industrially important acid dyes used in textile industry. Ammonium sulphate precipitated proteins from turnip root were taken for the treatment of a number of acid dyes. Partially purified preparation of TP was obtained by adding 20-80% ammonium sulphate and this preparation exhibited a specific activity of 122.0 U mg^{-1} protein. The experiments were designed to investigate the dye decolorization in the presence of H_2O_2 and partially purified TP. The dye solutions were found to be stable upon exposure to H_2O_2 or enzyme alone.

Decolorization of various acid dyes by TP was studied by varying incubation conditions of the reaction mixture like enzyme concentration, time, pH and temperature. It was evident that 1 h of reaction time was sufficient for maximum dye removal (Table 11). Recently, Mohan et al. (2005) have demonstrated decolorization of Acid Black 10 BX by HRP and dye decolorization was maximum in 45 min.

Decolorization of textile reactive dyes in the absence of HOBT was followed by the formation of precipitate, which settled down and could be removed by centrifugation. Several earlier investigators have shown that the treatment of phenols and aromatic amines by peroxidases and tyrosinases resulted in the formation of large insoluble aggregates (Husain and Jan, 2000; Duran and Esposito, 2000). However, decolorization

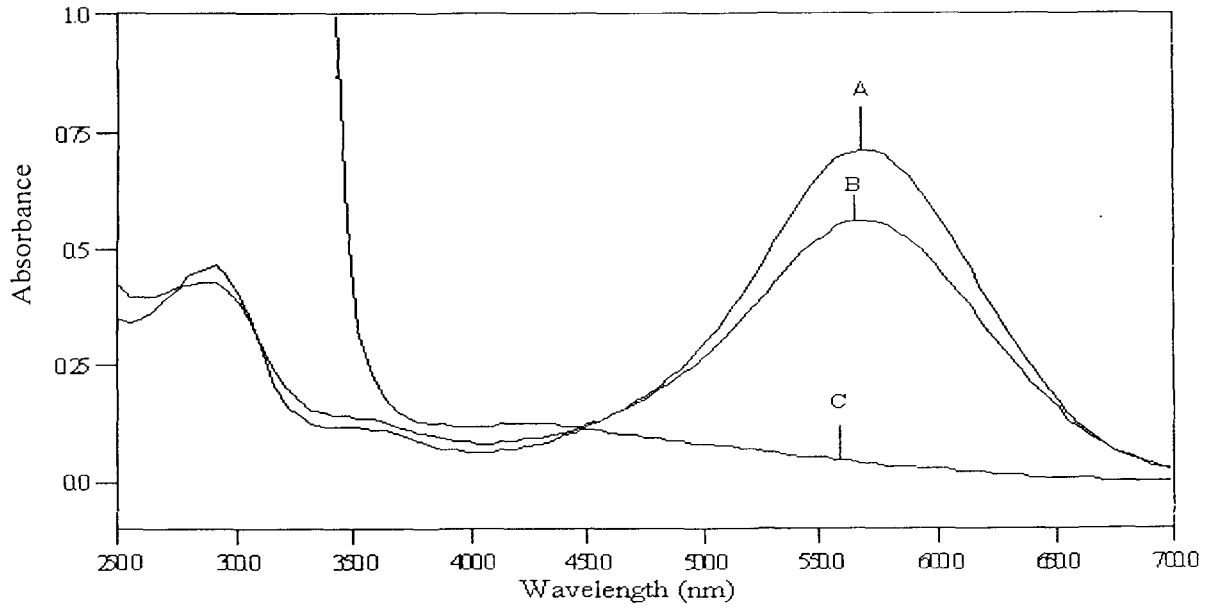

Figure 17: UV-Visible absorption spectra of Acid Blue 92

Acid Blue 92 was incubated with TP (0.235 U mL^{-1}) and 0.75 mM H_2O_2 in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h in the presence and absence of 2.0 mM HOBT. Spectra for the control, and TP treated dye solution were taken on Cintra 10*e* UV-visible spectrophotometer. Untreated dye (A), treated dye in the absence of HOBT (B) and in the presence of HOBT (C).

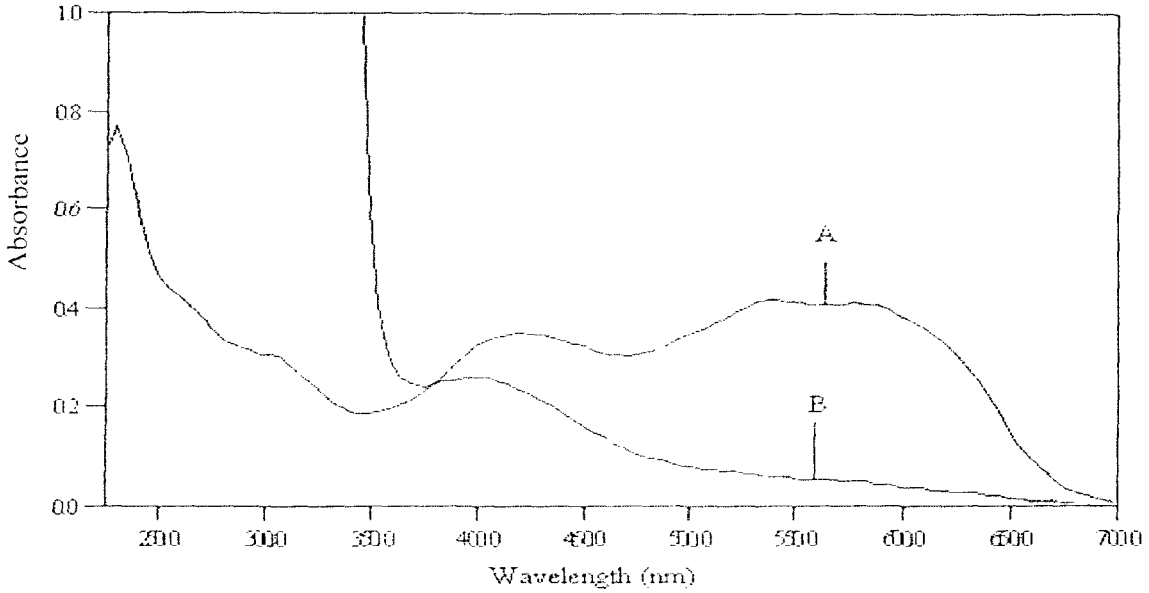

Figure 18: UV-Visible absorption spectra of a mixture of dyes

The dye mixture (Acid Red 97, Acid Yellow 42, Acid Blue 92 and Acid Black 1) was incubated with TP (0.235 U mL^{-1}) and 0.75 mM H_2O_2 in 100 mM sodium acetate buffer, pH 5.0 at 37 °C for 1 h in presence of 2.0 mM HOBT. Spectra for the control, and TP treated dye mixture were taken on Cintra 10*e* UV-visible spectrophotometer. Untreated dye mixture (A) and treated dye mixture in the presence of HOBT (B).

of acid dyes by TP in the presence of 2.0 mM HOBT appeared without the formation of any precipitate. It suggested that the decolorization of dyes took place via degradation of aromatic ring of the compounds or by cleaving certain functional groups. These results were in agreement with earlier published findings (Bhunia et al., 2001; Shaffiqu et al., 2002; Akhtar et al., 2005a). Hence, HOBT could have a dual role, first as a mediator in increasing the substrate range of dyes for TP and second enhancing the rate of oxidation of various substrates.

The decolorization of all the acid dyes by TP was optimum in the buffer of pH 5.0 and at 40 °C (Figs. 15,16). In an earlier study, it has been shown that HRP and BGP could decolorize and degrade dyes maximally at pH 2.5 and 3.0, respectively (Bhunia et al., 2001; Akhtar et al., 2005b). Temperature-optima was in agreement with an earlier reported decolorization of reactive dyes by BGP (Akhtar et al., 2005b).

To simulate the decolorization of dyes from industrial effluent, complex mixtures of various acid dyes were prepared and treated with TP (Table 12). However, the rate of decolorization of mixtures of dyes was slower than that of pure dye solution. This supports an earlier observation that the biodegradation of various phenols in the form of mixtures was quite slow as compared to an independent phenol (Kahru et al., 2000).

The individual dye solutions and their mixtures exhibited a significant loss in the TOC content when treated with TP at pH 5.0 (Tables 15 and 16), which was an important signal for the detoxification of aromatic compounds from wastewater. Some authors have reported a significant loss of TOC from the peroxidase treated dye solutions (Akhtar et al., 2005b; Matto and Husain, 2007).

The decrease in absorbance peaks in visible region was clear evidence regarding the removal of dyes from TP treated wastewater containing complex mixtures of dyes (Figs. 17,18). These spectral measurements provided strong evidences for the removal of aromatic pollutants from wastewater. The disappearance of absorption peak in the presence of HOBT in visible region was due to the breakdown of chromophoric groups present in the dyes (Bhunia et al., 2001). The decolorization of Direct Fast Scarlet 4BS in the microbial consortium composed of Pseudomonas 1-10 and White-rot fungus 8-4* also indicated the formation of intermediates with phenyl ring as the major components in the UV region, and its content was suggested to be more than in the original solution.

The absorbance peaks appeared in the UV region after TP treatment was quite comparable to the results explained by Fang et al. (2004).

Bourbonnais and Paice (1990) described for the first time the use of redox mediators by allowing laccase to oxidize non-phenolic compounds thereby expanding the range of substrates that can be oxidized by this enzyme. The mechanism of action of laccase-mediator system has been extensively studied and it is used in textile industry in the finishing process for indigo stained materials. Several workers have demonstrated that use of enzyme/redox mediator system enhanced the rate of dye decolorization by several folds but these mediators were required in very high concentrations (5.7 mM violuric acid/laccase system, 11.6 mM of HOBT/laccase system) (Soares et al., 2001; Claus et al., 2002). In this study, for the first time, we have shown decolorization of dyes by TP by using very low concentration of HOBT (2.0 mM), which enhanced the rate of acid dyes decolorization by 5-92 folds. Most recently, Akhtar et al. (2005a) have demonstrated the use of redox-mediator in the decolorization of dyes by using a peroxidase from bitter gourd. Our results further support that peroxidases from other sources could also be used for the decolorization of recalcitrant dyes in the presence of redox mediators.

The potential application of TP for dye degradation was tested using a number of chemically diverse commercially available acid dyes. Dye solutions, were successfully treated with TP in the presence and absence of redox mediator, HOBT. Decolorization rate was drastically increased when industrially important acid dyes were treated with TP in the presence of 2.0 mM HOBT. In order to understand the application of TP in effluent treatment, we prepared complex mixtures of dyes. The complex mixtures of dyes treated with TP in the presence of HOBT were significantly decolorized. The application of peroxidase that is easily available and inexpensive can overcome its limitations in wastewater treatment. The use of peroxidases can be extended to the large-scale treatment of a wide spectrum structural dye by using immobilized TP and relatively cheaper redox-mediators. This as well as the scale up of enzymatic processes is the subject of further study.

CHAPTER-V

Decolorization and Decontamination of Textile Carpet Industrial Effluent by Using Immobilized Turnip (*Brassica rapa*) and Tomato (*Lycopersicon esculentum*) Peroxidases

5.1. INTRODUCTION

Treatment of dye-based effluents is considered to be one of the challenging tasks in environmental fraternity. Even though physico-chemical methods are effective in the removal of dyes, the overall cost, regeneration problem, secondary pollutant/sludge generation limits their usage (Robinson et al., 2001b; Husain, 2006; Hai et al., 2007). However, the biological procedures have their own limitations such as the non-biodegradability of the xenobiotic compounds due to lack of requisite enzymes in the biological treatment plant (Robinson et al., 2001b; Keharia and Madamwar, 2003). Often the environment of the microorganisms is not optimal for rapid degradation of pollutants (McMullan et al., 2001; Robinson et al., 2001b). Researchers have been focusing their attention to study enzymatic pretreatment as a potential and viable alternative to conventional methods, due to its highly selective nature. Further inhibition by toxic substances is minimum in enzymatic treatment and the process can operate over a broad aromatic concentration range with low retention time (Karam and Nicell, 1997). The catalytic action of enzymes is extremely efficient and selective compared to chemical catalysts due to higher reaction rates, milder reaction conditions and greater stereospecificity.

There is an increasing demand in the study of peroxidases due to their action on wide spectrum aromatic pollutants. Enzymes from various sources have been applied for the treatment of aromatic compounds particularly dyes (Novotny et al., 2001). Peroxidases from fungal sources have proved their potential in the degradation and decolorization of synthetic dyes (Manimekalai and Swaminathan, 2000; Novotny et al., 2001; Maximo and Costa-Ferreira, 2004; Hou et al., 2004). There are also some reports about the use of plant peroxidases in the removal of pollutants from wastewater (Koller et al., 2000; Bhunia et al., 2001; Akhtar et al., 2005a; 2005b; Akhtar and Husain, 2006). The major reason that enzymatic treatments have not yet been applied on an industrial scale is the huge volume of polluted wastewater demanding remediation.

Several limitations prevent the use of free enzymes as the stability and catalytic ability of free enzymes decrease with the complexity of the effluents (Zille et al., 2003; Husain, 2006). Some of these limitations could be overcome by the use of enzymes in

immobilized form that would be used as catalysts with long lifetime (Rogalski et al., 1995; Zille et al., 2003). Enzyme immobilization procedures often require purified enzyme and expensive supports or reagents (Levy et al., 2003) that make immobilized enzyme preparation more expensive. Bioaffinity based procedures are receiving attention due to immobilization of enzymes even from the crude homogenate or partially purified enzyme preparation (Akhtar et al., 2005c; Khan et al., 2005; Matto and Husain, 2006).

In this study, the role of Con A-cellulose immobilized turnip and tomato peroxidases in the degradation/decolorization of two textile carpet industrial effluents has been investigated. Both textile effluents were decolorized/degraded by turnip/tomato peroxidases in the presence of 2.0 mM HOBT. The effect of enzyme concentration, time of incubation, pH and temperature has been investigated on the decolorization of two textile effluents; textile carpet effluent red (TCER) and textile carpet effluent blue (TCEB). The reusability of immobilized peroxidase preparations in the decolorization of effluent was also examined.

5.2. MATERIALS AND METHODS

5.2.1. Materials

Untreated textile carpet industrial effluents were collected from a carpet industry 'Ohm International' located in textile industrial area of Hathras (UP), India. Ammonium sulphate and HOBT were purchased from SRL Chemicals, Mumbai, India. *o*-dianisidine-HCl was obtained from IGIB, New Delhi, India. Cellulose powder and jack bean meal were procured from, Centron Research Labs, Mumbai and Loba Chem Co. India, respectively. Turnip and tomato were purchased from a local vegetable market. The chemicals and other reagents employed were of analytical grade and were used without any further purification.

5.2.2. Ammonium sulphate fractionation of turnip and tomato proteins

Turnip (50 g) and tomato (100 g) were homogenized independently in 100 mL of 100 mM sodium acetate buffer, pH 5.5. Homogenates were filtered through four layers of cheesecloth. The filtrates were then centrifuged at 10,000 *g* on a Remi Cooling Centrifuge C-24. The clear supernatants thus obtained were subjected to salt fractionation by adding 20-80% (w/v) $(NH_4)_2SO_4$. The mixtures were stirred overnight at 4 °C to obtain maximum precipitates. The precipitates were collected by centrifugation at 10,000 *g* on a Remi Cooling Centrifuge C-24. The obtained precipitates were re-dissolved in appropriate volume of 100 mM sodium acetate buffer, pH 5.5 and dialyzed against the assay buffer (Matto and Husain, 2006).

5.2.3. Immobilization of turnip and tomato peroxidases on Con A-cellulose support

Immobilization of TP and TMP on Con A-cellulose support was made according to the procedure described in chapter-III.

5.2.4. Treatment of textile effluents by increasing concentrations of peroxidases

Textile effluents were diluted in 100 mM sodium acetate buffer, pH 5.5. Each effluent was incubated with increasing concentrations of TP/TMP (0.141-0.849 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.5 in the presence of 0.75 mM H_2O_2 for 1 h at 37 °C. HOBT (2.0 mM) was used as a redox mediator for all the experiments. Decolorization of effluents by TP/TMP was monitored at their respective wavelength maxima in the presence and absence of 2.0 mM HOBT. The percent decolorization was calculated by taking untreated effluent as control (100%).

5.2.5. Treatment of textile effluents by peroxidases for varying times

Each textile effluent was incubated with TP (0.423 U mL^{-1}) and TMP (0.705 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.5 at 37 °C in the presence of 0.75 mM H_2O_2 for varying time intervals; 15 min to 2 h. Decolorization was also performed in the presence of 2.0 mM HOBT under other similar experimental conditions. The decrease in absorbance was monitored at predetermined intervals at the respective λ_{max} of each effluent. The percent decolorization was calculated by taking untreated effluent as control (100%).

5.2.6. Effect of pH on the decolorization of textile effluents by peroxidases

The textile effluents were treated by soluble and immobilized TP (0.423 U mL^{-1}) and TMP (0.705 U mL^{-1}) in the buffers of various pH in the presence of 0.75 mM H_2O_2 and 2.0 mM HOBT for 1 h at 37 °C. Decolorization of effluents by soluble and immobilized TP/TMP was monitored at their specific wavelength maxima. The percent decolorization was calculated by taking each untreated effluent in respective buffer as control (100%).

5.2.7. Effect of temperatures on the decolorization of textile effluents by peroxidases

Each textile effluent was incubated with soluble and immobilized TP (0.423 U mL^{-1}) and TMP (0.705 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.5 in the presence of 0.75 mM H_2O_2 and 2.0 mM HOBT at 30-80 °C for 1 h. Decrease in color of textile effluents after treatment with soluble and immobilized peroxidase was monitored at each specific wavelength. The percent decolorization was calculated by taking untreated effluent incubated at indicated temperature as control (100%).

5.2.8. Determination of TOC content of control and treated textile effluents

Each textile effluent was incubated with soluble and immobilized TP (0.423 U mL^{-1}) and TMP (0.705 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.5 in the presence of 0.75 mM H_2O_2 and 2.0 mM HOBT for 1 h at 37 °C. The effluents treated with soluble TP/TMP were kept in boiling water bath for 5 min to stop the reaction. The immobilized TP/TMP was separated from the treated reaction mixture by centrifugation at 3000 *g* for 15 min. The soluble and immobilized TP/TMP treated effluents were further incubated at 37 °C for 20 h. On the next day the treated effluents were centrifuged at 3000 *g* for 15 min to remove insoluble product. After centrifugation, the TOC content of the clear supernatant was determined by using a total organic carbon analyzer (Multi N/C 2000, Analytic Jena, Germany). Untreated textile effluent was considered as control. Both controls and treated effluents were diluted to 10-fold before measuring TOC content.

5.2.9. UV-visible spectral analysis

Procedure for the effluent decolorization was followed by the UV-visible spectral analysis. Spectra for the control, soluble and immobilized TP/TMP treated effluents were taken on Cintra 10*e* UV-visible spectrophotometer.

5.2.10. Reusability of Con A-cellulose bound peroxidases in the decolorization of textile effluents

The reusability of Con A-cellulose bound peroxidase preparations in the decolorization of textile effluents was monitored. Each textile effluent was incubated with immobilized TP (0.423 U mL^{-1}) and TMP (0.705 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.5 in the presence of 0.75 mM H_2O_2 and 2.0 mM HOBT for 1 h at 37 °C. After incubation time, immobilized enzyme was separated by centrifugation and stored in assay buffer for overnight at 4 °C. Such experiments were repeated eight times with the same aliquot of immobilized TP and TMP under the specified conditions by taking fresh batch of textile effluent. Decrease in color of textile effluents after treatment

with immobilized peroxidase preparations was monitored at specific wavelength maxima for both effluents. The percent decolorization was calculated by taking each untreated effluent as control (100%).

5.2.11. Measurement of peroxidase activity

Peroxidase activity was estimated according to the procedure described in chapter-II.

5.2.12. Determination of protein concentration

The protein concentration was determined according to the procedure described in chapter-II.

5.3. RESULTS

5.3.1. Treatment of textile effluents with increasing concentrations of peroxidases

Each textile effluent was incubated with increasing concentrations of TP/TMP "0.141-0.849 U mL^{-1}" in 100 mM sodium acetate buffer, pH 5.5 in the presence of 0.75 mM H_2O_2 and 2.0 mM HOBT for 1 h at 37 °C. Decolorization of textile effluents was continuously enhanced by adding increasing concentration of TP/TMP. TP was proved to be more efficient in the decolorization of textile effluent as only 0.423 U mL^{-1} of TP was sufficient for the maximum of decolorization of both effluents. However, the effluents treated by TMP required higher concentration for decolorization (Fig. 19). TCER and TCEB were decolorized 75% and 80% by TP (0.423 U mL^{-1}), whereas these effluents were decolorized 69% and 59% by TMP (0.705 U mL^{-1}), respectively.

5.3.2. Treatment of textile effluents with peroxidases for varying times

Textile effluents were incubated with TP (0.423U mL^{-1}) and TMP (0.705 U mL^{-1}) for varying times. Figure 20 demonstrates that both enzymes were quite effective in

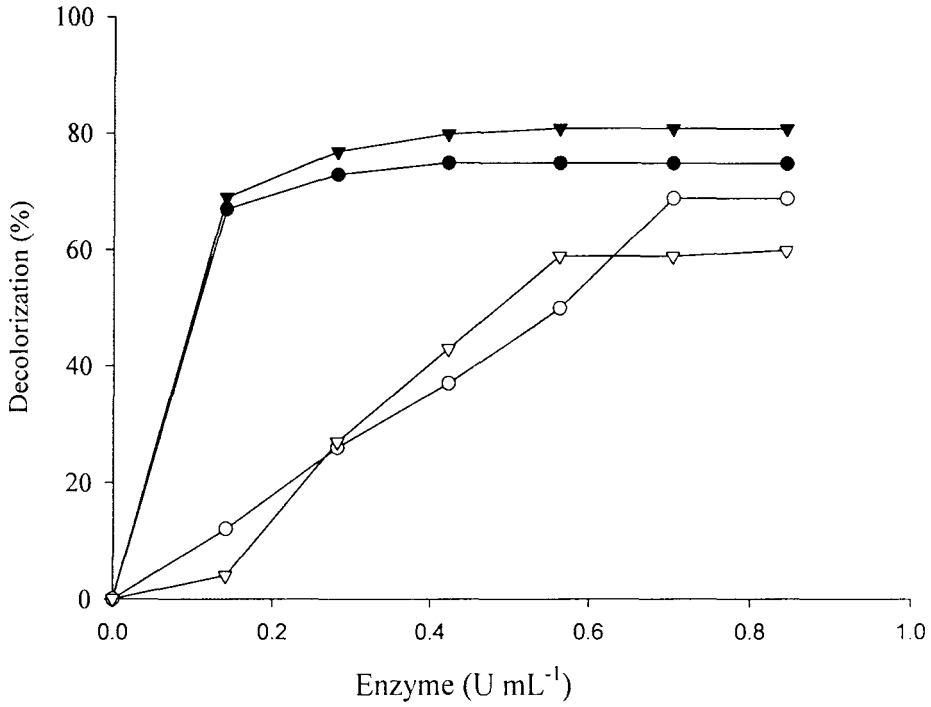

Figure 19: Effect of TP/TMP concentration on the decolorization of textile effluents

Each textile effluent was treated with increasing concentration of STP/STMP "0.141-0.849 U mL^{-1}" in 100 mM sodium acetate buffer, pH 5.5 in the presence of 0.75 mM H_2O_2 and 2.0 mM HOBT for 1 h at 37 °C. Symbols indicate STP (•) and STMP (○) decolorized TCER, and STP (▼) and STMP (▽) decolorized TCEB.

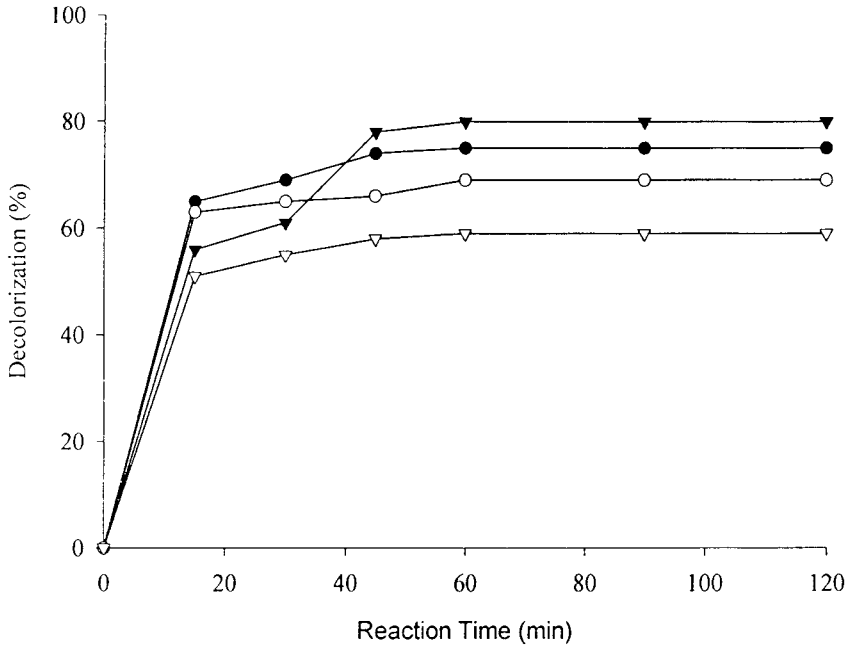

Figure 20. Decolorization of textile effluents by STP/STMP for varying times

Each textile effluent was treated with STP (0.423 U mL^{-1}) and STMP (0.705 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.5 at 37 °C in the presence of 0.75 mM H_2O_2 and 2.0 mM HOBT for varying time intervals; 15 min to 2 h Symbols indicate STP (•) and STMP (○) decolorized TCER, and STP (▼) and STMP (▽) decolorized TCEB.

catalyzing the decolorization of textile effluents. TCER and TCEB were decolorized 65% and 56% by soluble TP, whereas these effluents were decolorized 63% and 51% by soluble TMP after 15 min, respectively. Although more color disappeared when textile effluents were incubated for longer duration, however the rate of decolorization was quite slow. After 1 h of the reaction time no more significant decolorization of the textile effluents was observed.

5.3.3. Effect of pH on the decolorization of textile effluents by peroxidases

Textile effluents were treated with equal amount of soluble and immobilized TP/TMP in the buffers of different pH (Table 17). Both effluents were maximally decolorized at pH 5.0 when treated with soluble and immobilized TP whereas the effluent decolorization maxima for TMP was at pH 6.0. As the pH of decolorizing sample was increased up to pH 8.0, the rate of decolorization decreased in all the treated effluents. TMP showed maximum decolorization over a wide range of pH (5.0-8.0). However, in alkaline range effluents were recalcitrant for the action of TP.

5.3.4. Effect of temperatures on the decolorization of textile effluents by peroxidases

The effect of different temperatures (30-80 °C) on the decolorization of textile effluents was monitored and it was observed that all the peroxidase preparations decolorized effluents maximally at 40 °C. Above and below temperature-optima, the rate of effluent decolorization was low. At higher temperatures immobilized enzyme preparations were found to be more efficient in the removal of higher percent of color from textile effluents as compared to their soluble counterparts (Table 18).

5.3.5. TOC content of TP/TMP treated textile effluents

Both textile effluents were treated with soluble and immobilized TP/TMP and their insoluble products were removed by centrifugation. TOC content of textile effluents before and after treatment with soluble and immobilized TP/TMP is given in Table 19.

Table 17: Effect of pH on the decolorization of textile effluents by soluble and immobilized TP/TMP

pH	Decolorization (%)							
	TCER				TCEB			
	STP	ITP	STMP	ITMP	STP	ITP	STMP	ITMP
3	3	6	0	9	19	37	0	11
4	70	72	59	68	54	75	43	62
5	75	78	66	72	80	84	54	70
6	48	54	69	73	24	50	59	74
7	19	33	67	69	17	34	57	68
8	3	11	48	56	0	4	52	64
9	0	0	32	47	0	0	25	57
10	0	0	16	28	0	0	19	36

Each textile effluent was treated with soluble and immobilized TP (0.423 U mL^{-1}) and TMP (0.705 U mL^{-1}) in buffers of different pH (3.0-10.0) at 37 °C for 1 h. HOBT (2.0 mM) was used as a redox-mediator. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5 %.

Table 18: Effect of temperature on the decolorization of textile effluents by soluble and immobilized TP/TMP

Temperature (°C)	Decolorization (%)							
	TCER				TCEB			
	STP	ITP	STMP	ITMP	STP	ITP	STMP	ITMP
30	69	72	59	68	69	82	50	64
40	76	78	69	73	80	84	59	74
50	52	62	62	70	63	80	53	70
60	44	56	53	66	52	74	40	54
70	39	53	41	51	40	67	31	45
80	37	50	28	39	32	46	16	35

Each textile effluent was treated with soluble and immobilized TP (0.423 U mL^{-1}) and TMP (0.705 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.5 at 30-80 °C for 1 h. HOBT (2.0 mM) was used as a redox-mediator. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5 %.

Table 19: TOC content of textile effluents treated with soluble and immobilized TP/TMP

Textile effluents	TOC content (ppm)	
	TCER	TCEB
Control	205	243
STP Treated	58	46
ITP Treated	39	37
STMP Treated	75	57
ITMP Treated	64	42

Each textile effluent was treated independently with soluble and immobilized TP (0.423 U mL^{-1}) and TMP (0.705 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.5 at 30-80 °C for 1 h. HOBT (2.0 mM) was used as a redox-mediator. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5 %.

As compared to control, the level of TOC was decreased in both the soluble and immobilized TP/TMP treated samples. However, the immobilized TP treated effluent exhibited significant loss of TOC from the solution. These observations suggested that major toxic compounds get easily removed out of the TP/TMP treated solutions.

5.3.6. UV-visible spectral analysis

Figure 21 summarizes the decolorization and degradation of TCEB by soluble and immobilized TP/TMP in the presence of 2.0 mM HOBT. This observation clearly demonstrates diminution in the absorbance peak in the visible region after the treatment with peroxidase preparations. However, the soluble and immobilized TP/TMP treated reaction mixture showed a very high absorbance at 350 nm in the UV region.

The immobilized TP/TMP treated reaction mixture exhibited significantly higher percent of color removal than the soluble TP/TMP. It was also observed that TP was more effective than TMP in the decolorization and degradation of textile effluents.

5.3.7. Reusability of Con A-cellulose bound peroxidases in the decolorization of textile effluents

In order to make immobilized preparation more practical in a reactor, the reusability of immobilized TP/TMP for the decolorization of textile effluents has been investigated. The effluent decolorizing reusability was continuously decreased up to the 8^{th} repeated use of the same preparation. Immobilized TP retained greater fractions of catalytic activity after 8^{th} use as compared to immobilized TMP (Table 20).

5.4. DISCUSSION

The decolorization of dyes by peroxidases is now well documented (Bhunia et al., 2001; Akhtar et al., 2005a; 2005b). In the present work, an effort has been made to treat textile carpet industrial effluents by using soluble and immobilized turnip and tomato peroxidases. In order to reduce the cost of the wastewater treatment, immobilization of

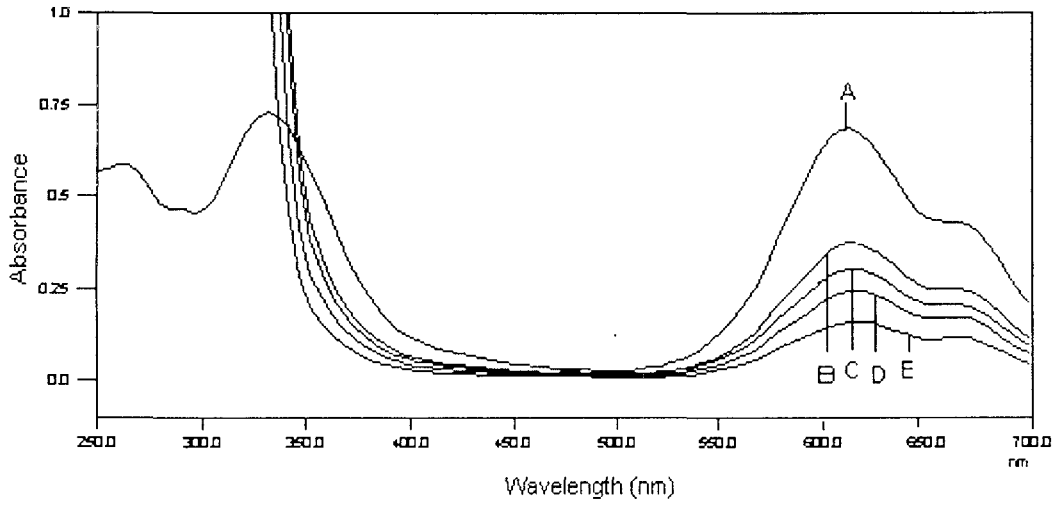

Figure 21: UV-Visible absorption spectra of textile carpet effluent blue (TCEB)

The textile effluent was treated with soluble and immobilized TP (0.423 U mL^{-1}) and TMP (0.705 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.5 at 37 °C for 1 h. HOBT (2.0 mM) was used as a redox-mediator in all the reactions. Spectra for the control and TP/TMP treated effluent were taken on Cintra 10*e* UV-visible spectrophotometer. The spectra labeled as (A) control; (B) STMP treated; (C) ITMP treated; (D) STP treated; (E) ITP treated effluent solution.

Table 20. Reusability of Con A-cellulose bound TP/TMP for the decolorization of textile effluents

Number of uses	Decolorization (%)			
	TCER		TCEB	
	ITP	ITMP	ITP	ITMP
1	76	72	83	71
2	75	70	81	68
3	71	66	75	59
4	65	51	69	55
5	56	43	62	47
6	50	26	51	34
7	45	21	47	29
8	41	18	40	24

Each textile effluent was incubated with immobilized TP (0.423 U mL^{-1}) and TMP (0.705 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.5 in the presence of 0.75 mM H_2O_2 and 2.0 mM HOBT for 1 h at 37 °C. Reusability was studied upto 8^{th} repeated use. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5 %.

turnip and tomato peroxidases have been carried out directly from ammonium sulphate fractionated proteins on Con A-cellulose. The dye effluents were found to be stable upon exposure to H_2O_2 or enzyme alone. Thus, the dye removal was a result of H_2O_2-dependent enzymatic reaction, possibly involving free-radical formation followed by polymerization and precipitation (Bhunia et al., 2001; Mohan et al., 2005).

The rate of dye decolorization was dependent on the concentration of enzyme and the time of incubation (Figs. 19,20). The results showed that STP preparation was more effective in the decolorization of industrial effluent as compared to STMP under various experimental conditions.

Initial first hour was found to be significant for the textile effluent decolorization. The increase in the rate of effluent decolorization was insignificant after 1 h, which might be due to the product inhibition. Our results were in agreement with earlier published work, which showed that 45 min and 1 h of incubation time was sufficient for maximum dye decolorization (Mohan et al., 2005; Akhtar et al., 2005a).

The use of free enzymes has several drawbacks such as the stability, reusability and catalytic efficiency due to complexity of the effluents (Zille et al., 2003). Some of these limitations can be overcome by the use of immobilized enzymes, which could be used as catalysts with long lifetime and in continuous reactors (Zille et al., 2003; Husain, 2006). Mohan et al. (2005) reported that acrylamide gel immobilized HRP was more effective in decolorizing Acid Black 10BX compared to alginate entrapped and free HRP.

Con A-cellulose adsorbed peroxidase preparations had very high stability against several forms of denaturants as mentioned in chapter-III. In view of its high stability and cost of preparation, it could be exploited for the treatment of textile effluents. The decolorization of textile effluents by TP and TMP was maximum at pH 5.0 and pH 6.0, respectively (Table 17). In an earlier study it has been reported that the decolorization of reactive textile dyes by soluble and immobilized BGP was maximum in the range of pH 3.0-4.0 (Akhtar et al., 2005b). Some recent studies have shown that direct dyes were decolorized maximally at pH 5.0 by TP (Matto and Husain, 2007). The decolorization of textile effluents by TP and TMP was maximum at 40 °C (Table 18).

The level of TOC was remarkably decreased in both soluble and immobilized peroxidase preparations treated textile effluents. However, Con A-cellulose bound

peroxidase preparations treated effluent exhibited significant loss of TOC (Table 19). Immobilized HRP has been shown to remove 88% of TOC from model wastewater containing mixture of chlorophenols (Tatsumi et al., 1996). Several earlier workers have confirmed the removal of dyes from wastewater in the form of TOC by treating them with certain chemical methods such as ozonation (Koch et al., 2002). Recently, some investigators have also reported significant loss of TOC from the peroxidase treated dye solutions (Akhtar et al., 2005b; Matto and Husain, 2007).

In order to confirm the decolorization and degradation of textile effluent from the TP/TMP treated reaction mixtures, the spectral analysis was also performed in the presence of 2.0 mM HOBT (Fig. 21). The diminution in absorption peak in visible region was due to the breakdown of chromophoric groups present in the textile effluent (Akhtar et al., 2005a). However, a very high absorbance at 350 nm in the UV region indicated the formation of intermediates with aromatic rings as the major components in the reaction mixture, and its content might be more than that in the control. Our spectral analysis was quite comparable to the UV-visible spectra of culture containing Direct Fast Scarlet 4BS after degradation by the microbial consortium composed of Pseudomonas 1-10 and White-rot fungus 8-4* (Fang et al., 2004).

The objective of immobilization is the reusability of the matrix in the process. The investigations were carried out to assess repeated usability of Con A-cellulose bound peroxidase for effluent decolorization. The effluent decolorizing reusability was continuously decreased up to the 8^{th} repeated use of the same preparation. This may be either due to leaching of enzyme from bioaffinity support in varying amounts or product inhibition (Table 20). Shaffiqu et al. (2002) have shown that hydrophobic matrix bound Saccharum peroxidase could effectively degrade four textile dyes. The immobilized enzyme was used in a batch reactor for the degradation of Procion Green HE-4BD and the reusability was studied for 15 cycles. In a recent study, Mohan et al. (2005) have investigated the repeated usability of entrapped HRP beads for an acid dye removal from wastewater.

This study indicates that an enzymatic approach has attracted much interest in the remediation/decolorization of various effluents collected from textile carpet industry. The treatment of such pollutants by plant peroxidases sometimes causes problems due to

recalcitrant nature of the compounds. Such recalcitrant compounds could be successfully decolorized by peroxidases in the presence of redox mediators. Immobilized peroxidases further have shown their potential over the soluble form. Immobilized enzymes could be successfully used in the reactors for continuous decolorization of dyes from effluents. Treatment of recalcitrant pollutants by using enzyme-redox mediator system will be feasible.

Summary

Enzymes have a great appeal in chemical processes as 'green chemistry' reagents that will allow future sustainable developments. In this sense, enzyme technology has become a multidisciplinary field with applications in diverse processes. Peroxidases in particular have been extensively studied and show many attractive properties for biocatalysis such as wide specificity, high stability in solution and easy accessibility from plant materials. Bitter gourd and turnip peroxidases have generated great interest due to its different activity and stability profile compared to its widely used counterpart, horseradish peroxidase. Immobilization is an efficient way to prevent inactivation and extend enzyme half-life, besides, immobilization procedures can increase structural rigidity thus improving pH, temperature and organic solvent tolerance.

In the present study, an effort has been made to immobilize peroxidases directly from ammonium sulphate precipitated proteins of bitter gourd on an anion exchanger, DEAE cellulose. The activated DEAE cellulose was quite effective in high yield immobilization of peroxidases from bitter gourd and it bound peroxidase activity 590 U g^{-1} of the matrix. Bitter gourd peroxidase immobilized on this anion exchanger showed very high effectiveness factor 'η' as 0.95. BGP could bind very strongly to the DEAE cellulose, as it did not detach even in the presence of 0.5 M NaCl. The incubation of immobilized enzyme with 0.1 M NaCl for 24 h resulted in a loss of only 13% of the initial activity. Immobilized bitter gourd peroxidase preparation was more stable to denaturation induced by pH, heat, urea, proteolytic enzyme, detergents; Surf Excel and Rin Powder, Triton X-100 and water miscible organic solvents; dioxane, dimethyl sulphoxide and *n*-propanol. Peroxidase adsorbed on the matrix exhibited very high resistance to proteolysis mediated by the trypsin treatment. DEAE cellulose bound bitter gourd peroxidase lost 45% of its initial activity after treatment with 2.5 mg mL^{-1} of trypsin for 1 h at 37 °C while the soluble enzyme lost nearly 65% of the initial activity under similar incubation conditions.

Turnip peroxidase is also a very attractive and cost-effective candidate for bioremediation because it is obtained from turnip roots which are easily available in northern part of India. In order to keep costs down, especially with respect to traditional chemical processes, several attempts have been made to find an enzymatic method that could be easily applied on an industrial scale. Concanavalin A is finding increasing

applications as a useful ligand in glycoenzyme immobilization. Concanavalin A adsorbed cellulose has been employed for the simultaneous purification and immobilization of glycoenzymes directly from ammonium sulphate fractionated proteins of turnip. The bioaffinity support was prepared simply by incubating cellulose powder with jack bean extract at 4 °C. Cellulose powder adsorbed 30 mg concanavalin A g^{-1} of the matrix. The obtained bioaffinity support was quite effective in high yield immobilization of peroxidase from turnip and it retained 672 U g^{-1} of the matrix. Turnip peroxidase immobilized on concanavalin A-cellulose support retained 80% of the initial activity. Turnip peroxidase bound very strongly to Con A-cellulose support as it did not detach even in the presence of 0.8 M NaCl. Immobilized turnip peroxidase preparation was compared with its soluble counterpart for the stability against various forms of chemical and physical parameters. Immobilized turnip peroxidase preparation was quite resistant against the denaturation mediated by pH, heat, urea, guanidinium-HCl, detergents; Surf Excel, cetyltrimethylammonium bromide, Triton X-100, Tween 20 and water-miscible organic solvents; dimethyl formamide, dioxane and *n*-propanol. Low concentrations of detergents like Surf Excel and cetyltrimethylammonium bromide enhanced the activity of soluble and immobilized turnip peroxidase. The activity of soluble and immobilized turnip peroxidase was also enhanced in the presence of lower concentrations of non-ionic detergents (0.2-1.0%, v/v) like Triton X-100, Tween 20. This enhancement in enzyme activity of immobilized turnip peroxidase was remarkably very high.

The role of partially purified turnip peroxidase for the treatment of acid dyes has been investigated. Turnip peroxidase was fractionated from the buffer extract by 20-80% $(NH_4)_2SO_4$ precipitation. Peroxidases from turnip roots were highly effective in decolorizing acid dyes having wide spectrum chemical groups. Dye solutions, containing 40-170 mg L^{-1}, were treated by turnip peroxidases (specific activity of 122.0 U mg^{-1} proteins). The parameters such as the effect of enzyme concentration, time, pH and temperature were standardized for the decolorization of acid dyes. Turnip peroxidase was able to decolorize most of the acid dyes in the presence of 2.0 mM 1-hydroxybenzotriazole. Increasing concentration of enzyme and time in the absence of 1-hydroxybenzotriazole did not influence dye decolorization. The rate of decolorization was significantly enhanced when 1-hydroxybenzotriazole was added to the decolorizing

solutions. In initial first hour, most of the dyes were maximally decolorized. The decolorization of all the used dyes was maximum at pH 5.0 and 40 °C. In order to prove the compatibility of enzyme in the treatment of industrial effluents, we have investigated the treatment of mixtures of dyes with partially purified turnip peroxidase. Complex mixtures of dyes were significantly decolorized when treated with enzyme in the presence of 1-hydroxybenzotriazole (2.0 mM). Phytotoxicity test based on *Allium cepa* root growth inhibition has shown that majority of the turnip peroxidase-treated dye product were less toxic than their parent dye. Kinetic parameters of the turnip peroxidase with various dyes showed that this enzyme has highest affinity for Acid Yellow 42. The polluted wastewater contaminated with single dye or mixtures of dyes were treated with enzyme and it resulted in a remarkable loss of total organic carbon content. This study demonstrates that the peroxidase/mediator system was an effective biocatalyst for the treatment of effluents coming out from textile, dye manufacturing, dyeing and printing industries or complex mixtures of dyes.

Decolorization and decontamination of two textile carpet industrial effluents was carried out by using Con A-cellulose bound turnip and tomato peroxidases. Both effluents were recalcitrant to the action of these plant peroxidases. However, the decolorization of effluents was enhanced in the presence of 2.0 mM HOBT. Industrial effluents; textile carpet effluent red and textile carpet effluent blue were decolorized 75% and 80% by soluble turnip peroxidase (0.423 U mL^{-1}) and 69% and 59% by soluble tomato peroxidase (0.705 U mL^{-1}), respectively. After 15 min, textile carpet effluent red and textile carpet effluent blue were decolorized 65% and 56% by turnip peroxidase; however, these effluents were decolorized 63% and 51% by tomato peroxidase. Both effluents were maximally decolorized at pH 5.0 by soluble and immobilized turnip peroxidase whereas the maximum decolorization by soluble and immobilized tomato peroxidase was found at pH 6.0. However, it was observed that both effluents were decolorized maximally at 40 °C by soluble and immobilized peroxidases. The immobilized turnip peroxidase treated effluent exhibited significant loss of total organic carbon content from the solution. These observations suggested that major toxic compounds get easily removed out of the turnip and tomato peroxidase treated samples. Immobilized peroxidase preparations have a marginal edge over the soluble enzyme in

decolorizing the textile effluents. The absorption spectra of treated and untreated textile effluent exhibited a marked difference in the absorbance in the visible region. The effluent decolorizing reusability was continuously decreased up to their 8^{th} repeated use. Immobilized turnip peroxidase showed over 40% decolorization even after its 8^{th} use. This study showed that turnip peroxidase was more efficient than tomato peroxidase in the decolorization and degradation of textile effluents.

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LIST OF PUBLICATIONS AND PRESENTATIONS

1. **Kulshrestha, Y.**, Husain, Q. Direct immobilization of peroxidase on DEAE cellulose from ammonium sulphate fractionated proteins of bitter gourd (*Momordica charantia*). Enzyme Microb. Technol. 2006; **38:** 470-477.

2. **Kulshrestha, Y.**, Husain, Q. Bioaffinity-based an inexpensive and high yield procedure for the immobilization of turnip (*Brassica rapa*) peroxidase. Biomol. Eng. 2006; **23:** 291-297.

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6. **Kulshrestha, Y.**, Gupta, P., Husain, Q., 2005. Stabilization of trypsin by sugars. Nat. Symp. Rec. Adv. Sci. Persp. Held at Dolphin, P.G. Inst. Biomed. Sci., Dehradun, U.A. (India) June 3-5, Abstract No. TS-2/P29.

7. **Kulshrestha, Y.**, Husain, Q., 2006. Simultaneous purification and immobilization of turnip (*Brassica rapa*) peroxidase on bioaffinity support. 75th Ann. Meet. Soc. Biol. Chemists (India) Dec. 8-11, Abstract No. PIII-84.

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Direct immobilization of peroxidase on DEAE cellulose from ammonium sulphate fractionated proteins of bitter gourd (*Momordica charantia*)

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Abstract

The direct immobilization of peroxidases on DEAE cellulose from ammonium sulphate fractionated proteins of bitter gourd has been investigated. The activated DEAE cellulose was quite effective in high yield immobilization of peroxidases from bitter gourd and it could bind nearly 590 enzyme units per g of the matrix. Bitter gourd peroxidase immobilized on this anion exchanger showed very high effectiveness factor 'η' as 0.95. BGP bound very strongly to the DEAE cellulose, as it did not detach even in the presence of 0.5 M NaCl. Immobilized bitter gourd peroxidase preparation was more stable to the denaturation induced by pH, heat, urea, proteolytic enzyme, detergents (Surf Excel and Rin powder), Triton X 100 and water-miscible organic solvents (dioxane, dimethyl sulphoxide and *n*-propanol). Peroxidase adsorbed on the matrix exhibited very high resistance to proteolysis mediated by the trypsin treatment. DEAE cellulose bound bitter gourd peroxidase lost 45% of its initial activity after treatment with 2.5 mg trypsin per ml of incubation mixture for 1 h at 37 °C while the soluble enzyme lost nearly 65% of the initial activity under similar incubation conditions.

Keywords: Adsorption; Direct; DEAE cellulose; Bitter gourd; Immobilization; Peroxidase; Stabilization; *n*-Propanol

1. Introduction

Recently, it has been reported that peroxidases can be used in the detoxification and biotransformation of several phenols, aromatic amines, biphenyls, bisphenols and dyes present in polluted wastewater/industrial effluents coming out from several industries [1–5]. The soluble enzyme cannot be exploited at the large-scale due to some inherent limitations to treat the huge volume of effluents. On the other hand, the immobilized enzyme has offered several advantages, such as enhanced stability, easier product recovery and purification, protection of enzymes against denaturants, proteolysis and reduced susceptibility to contamination. Several methods have been used for the immobilization of peroxidases from various sources but most of the immobilized enzyme preparations either use commercially available enzyme or expensive supports [6,7]. These factors were affecting the cost of the immobilized enzyme system [8]. These expensive immobilized systems could not meet the requirements for the treatment of huge volume of effluents. However, among the techniques used for the immobilization of enzymes, adsorption on the insoluble supports has several merits over the other known methods. It is a simple procedure and can be exploited for the direct immobilization of enzymes even from the crude cell homogenates.

Adsorption procedures are significantly useful for the immobilization of enzymes directly from the crude homogenate and thus avoiding the high cost of enzyme purification [9,10]. Ease of immobilization, lack of chemical modification and usually accompanying enhancement in stability are some of the advantages offered by the adsorption procedures. Several investigators have described the immobilization of enzymes on the bioaffinity supports for the specific

Abbreviations: BGP, bitter gourd peroxidase; DEAE, diethyl aminoethyl; DMSO, dimethyl sulphoxide

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immobilization of enzymes directly from partially purified enzyme preparation but these procedures require the use of expensive ligands, such as antibodies or lectins [6,11,12]. In order to minimize the cost of immobilization, the adsorption of proteins directly from partially purified enzyme preparation on an anion exchanger can be performed. These supports have already appeared in high yield and stable immobilization of enzymes.

Here, an effort has been made to immobilize the peroxidases on an anion exchanger, diethyl aminoethyl (DEAE) cellulose directly from the salt fractionated proteins of bitter gourd and dialyzed bitter gourd proteins. DEAE cellulose adsorbed bitter gourd peroxidase (BGP) preparation was compared with its soluble counter part for its stability against pH, heat, urea, detergents, water-miscible organic solvents and proteolytic enzyme (trypsin). DEAE cellulose adsorbed BGP preparation was significantly stable against several tested physical and chemical parameters.

2. Materials and methods

2.1. Materials

DEAE cellulose 11 was the product of SRL Chemicals, Mumbai, India. *o*-Dianisidine-HCl was purchased from the Center for Biochemical Technology, New Delhi, India. Ammonium sulphate, dioxane, dimethyl sulphoxide, *n*-propanol and Triton X 100 were obtained from the SRL Chemicals Mumbai, India. Surf Excel and Rin powder were purchased from the local market. Other chemicals and reagents employed were of analytical grade and were used without any further purification.

2.2. Ammonium sulphate fractionation of bitter gourd proteins

Bitter gourd (50 g) was homogenized in 100 ml of 50 mM sodium acetate buffer, pH 5.6. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000 × *g* on a Remi R-24 cooling centrifuge for 10 min at 4 °C. The clear supernatant was subjected to salt fractionation by adding 20–80% (w/v) $(NH_4)_2SO_4$. It was stirred overnight at 4 °C and the obtained precipitate was collected by centrifugation at 10,000 × *g* on a Remi R-24 cooling centrifuge for 10 min at 4 °C [6]. The collected precipitate was redissolved in 50 mM sodium acetate buffer, pH 5.6 and dialyzed against the assay buffer.

2.3. Activation of DEAE cellulose

DEAE cellulose (5.0 gm) was added to 100 ml of distilled water and was stirred slowly, kept overnight for swelling. Swelled DEAE cellulose was filtered on a Buchner funnel and was incubated with 100 ml of 0.5N HCl for 1 h. It was collected by filtration on Buchner funnel and was washed with distilled water continuously till it attained pH 7.0. Hundred milliliters of 0.5N NaOH was added to HCl treated DEAE cellulose and it was stirred on a magnetic stirrer for 1 h at room temperature 25 °C. It was washed again with distilled water till it attained neutral pH. Further, it was suspended and stored in 100 ml of distilled water at 4 °C.

2.4. Adsorption of BGP on activated DEAE cellulose

BGP (5535 units) was added to 5.0 g of DEAE cellulose and stirred overnight at 4 °C. Unbound BGP was removed by extensive washing with the assay buffer.

2.5. Effect of ion concentrations on the DEAE cellulose adsorbed BGP

The adsorbed BGP preparation was incubated with increasing concentration of NaCl (0.1–1.0 M) in 0.1 M sodium acetate buffer, pH 5.6 for 1 h at 37 °C. In order to monitor the effect of long-time exposure of immobilized enzyme with ions, the adsorbed BGP was also incubated with 0.1 M NaCl upto 24 h.

2.6. Effect of trypsin mediated proteolysis on the activity of soluble and immobilized BGP

Soluble and immobilized BGP preparations (1.25 units) were incubated with 0.25–2.5 mg trypsin/ml of incubation mixture at 37 °C for 1 h [6]. The activities of soluble and immobilized BGP in assay buffer without any trypsin treatment were taken as control (100%), for the calculation of percent activity. Peroxidase activity was determined according to the standard procedure.

2.7. Effect of Surf Excel and Rin powder on the activity of soluble and DEAE cellulose adsorbed BGP

Soluble and immobilized BGP preparations (1.25 units) were incubated independently with varying concentration of Surf Excel and Rin powder (0.1–1.0%, w/v) in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated detergent concentrations and other assay conditions were same as mentioned in the text. The activity of soluble and immobilized BGP in assay buffer without any detergent were taken as control (100%), for the calculation of percent activity.

2.8. Treatment of soluble and immobilized BGP with Triton X 100

Soluble and immobilized BGP (1.25 units) preparations were incubated with increasing concentration of Triton X 100 (0.5–5%, v/v) in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. After exposure the peroxidase activity was determined according to the procedure described in the text.

2.9. Effect of water-miscible organic solvents on the activity of soluble and immobilized BGP

Soluble and immobilized BGP preparations (1.25 units) were incubated with 10–60% (v/v) of water-miscible organic solvents; dioxane/dimethyl sulphoxide (DMSO)/*n*-propanol in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was determined at all the indicated organic solvent concentrations after the incubation period. Other assay conditions were the same as described in the text.

2.10. Measurement of peroxidase activity

Peroxidase activity was estimated from the change in the optical density (A_{460}, nm) at 37 °C by measuring the initial rate of oxidation of *o*-dianisidine–HCl in the presence of hydrogen peroxide, using both the substrates in saturating concentrations. The immobilized preparation was continuously agitated for the entire duration of assay. The assay was highly reproducible with immobilized preparations [7].

One unit of peroxidase activity was defined as the amount of enzyme protein that catalyzes the formation of 1 μmol of oxidized product of *o*-dianisidine–HCl in the presence of hydrogen peroxide per minute at 37 °C.

2.11. Determination of protein concentration

The protein concentration was determined by the procedure of Lowry et al. [14]. Bovine serum albumin was used as standard.

3. Results

3.1. Adsorption of BGP on DEAE cellulose

DEAE cellulose is commonly used for the purification and immobilization of variety of enzymes and proteins. In view of DEAE cellulose property to adsorb proteins on the basis of ionic interactions, this property has been exploited for directly binding the enzymes from the dialyzed salt fractionated bitter gourd proteins by simply incubating DEAE cellulose with ammonium sulphate fractionated dialyzed proteins overnight at 4 °C. After binding proteins on DEAE cellulose, the complex was washed with assay buffer till the traces of unbound proteins were removed. Unbound proteins were removed by extensive washing with assay buffer. DEAE cellulose adsorbed 590 units of peroxidase per g of the matrix. The effectiveness factor 'η' of the immobilized enzyme preparation was 0.95 (Table 1). High effectiveness factor of immobilized bitter gourd peroxidase preparation suggested that immobilized preparation was quite porous and effective in catalysis.

Table 1
Adsorption of BGP on DEAE cellulose

Amount of Enzyme loaded (X) (units)	1107
Amount of Enzyme activity in washes (Y) (units)	482
Activity bound/g of DEAE cellulose (units)	
Theoretical ($X - Y = A$) (A)	625
Actual (B)	590
Effectiveness factor (η) (B/A)	0.95
% of activity yield ($B/A \times 100$)	95

Each value represents the mean for three-independent experiments performed in duplicate, with average standard deviation <5%. Peroxidase activity was assayed according to the procedure given in the text.

3.2. Effect of ion concentrations on the DEAE cellulose adsorbed BGP activity

The wastewaters may also contain several types of ions; therefore it was necessary to examine the detachment of BGP from its support in the presence of various concentrations of ions. The exposure of adsorbed enzyme with increasing concentration of NaCl (0.1–1.0 M) for 1 h exhibited retention of very high enzyme activity even in the presence of 1.0 M NaCl. The incubation of immobilized BGP upto 0.5 M NaCl for 1 h had no detachment of enzyme activity (Fig. 1). In order to monitor the effect of long exposure of immobilized enzyme with ions, the adsorbed BGP was incubated with 0.1 M NaCl upto 24 h. The incubation of immobilized enzyme with 0.1 M NaCl for 24 h resulted in a slight loss of only 13% of the initial activity (data not given). These observations suggested that the binding of BGP with DEAE cellulose was quite strong and such type of immobilized enzyme preparations can be easily exploited for its use in the treatment of wastewater containing aromatic pollutants.

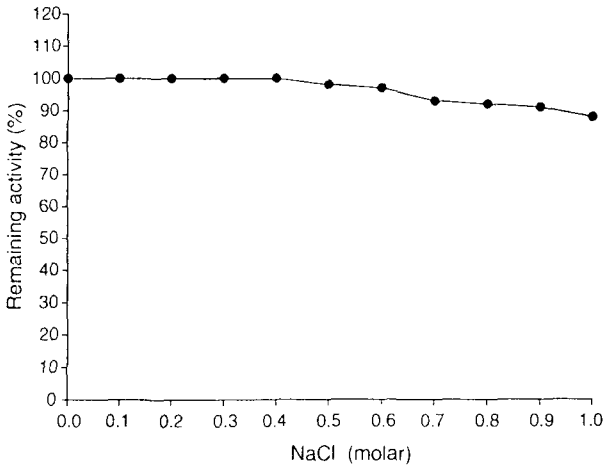

Fig. 1. Effect of ion (salt) concentration on the DEAE cellulose adsorbed BGP. DEAE cellulose adsorbed BGP preparation was incubated with increasing concentrations of NaCl (0.1–1.0 M) in 0.1 M sodium acetate buffer, pH 5.6 for 1 h at 37 °C. After incubation period each treated enzyme preparation was centrifuged to monitor the remaining adsorbed enzyme activity. Symbol (●) indicates immobilized enzyme.

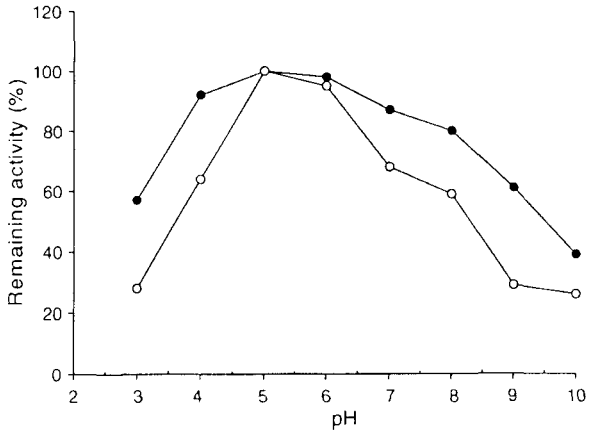

Fig. 2. pH–activity profiles of soluble and DEAE cellulose bound BGP. The appropriate amounts of soluble and immobilized BGP were taken for the preparation of pH–activity profile. The reaction mixture was incubated at 37 °C for 15 min in buffers of pH ranges from 3.0 to 10.0. The buffers used were 50 mM glycine–HCl for the pH 3.0, sodium acetate for the pH 4.0, 5.0, sodium phosphate for the pH 6.0, 7.0, 8.0 and Tris–HCl for the pH 9.0, 10. Symbols indicate the soluble (○) and immobilized (●) enzyme.

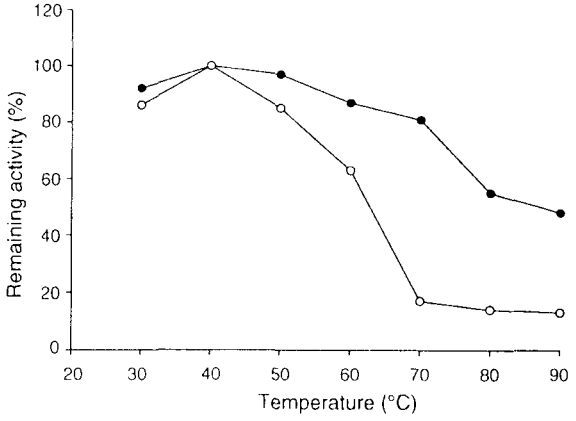

Fig. 3. Temperature–activity profiles of soluble and DEAE cellulose bound BGP. The activity of soluble and immobilized BGP (1.25 units) was monitored at various indicated temperatures. Activity expressed at 40 °C was taken as control for calculating percent activity. For symbols refer to Fig. 2.

3.3. Stability properties of soluble and DEAE cellulose bound BGP preparations

The stability of soluble and DEAE cellulose bound BGP preparations was monitored against various physical and chemical parameters because these parameters can affect the activity of the enzymes used for the treatment of organic pollutants present in the wastewater.

3.3.1. pH–activity profiles of soluble and DEAE cellulose bound BGP preparations

DEAE cellulose bound BGP showed broadening in the pH–activity profile as compared to the native enzyme (Fig. 2). Immobilized enzyme retained significantly higher enzyme activity on both sides of pH-optimum in comparison to free enzyme. pH-optimum of immobilized enzyme had no difference from pH 5.0 to 6.0, although soluble enzyme showed pH-optimum at pH 5.0.

3.3.2. Temperature–activity profiles of soluble and DEAE cellulose bound BGP preparations

DEAE cellulose bound BGP preparation had no change in temperature-optima as compared to its soluble counter part. Both the preparations exhibited temperature-optima at 40 °C. However, DEAE cellulose bound BGP retained significantly greater fractions of catalytic activity at high temperatures (Fig. 3).

3.3.3. Thermal denaturation plot of soluble and DEAE cellulose bound BGP preparations

Fig. 4 demonstrates the thermal denaturation of soluble and immobilized BGP at 60 °C for 2 h. Soluble BGP incubated at 60 °C for 2 h retained 43% of its initial enzyme activity while the immobilized enzyme incubated under similar conditions was significantly more stable to heat inactivation. The immobilized BGP exhibited 60% of the original activity after 2 h of heat treatment.

3.3.4. Urea mediated denaturation of soluble and DEAE cellulose bound BGP preparations

DEAE cellulose bound BGP was more resistant to inactivation induced by 4.0 M urea compared to its soluble counterpart. Exposure of soluble enzyme with 4.0 M urea for 2 h resulted in the loss of 53% activity whereas the immobilized enzyme retained more than 70% of the initial enzyme activity (Fig. 5).

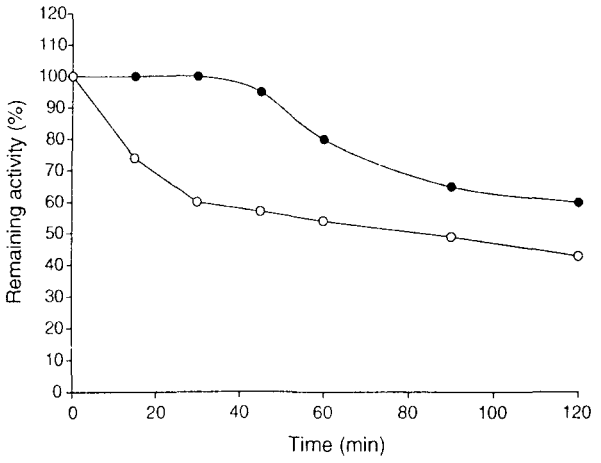

Fig. 4. Thermal denaturation of soluble and DEAE cellulose adsorbed BGP. Soluble and immobilized BGP (1.25 units) were incubated at 60 °C for varying times in 50 mM sodium acetate buffer, pH 5.6. Aliquots of each preparation were taken out at indicated time intervals and enzyme activity was determined as described in the text. Un-incubated samples at 60 °C were taken as 100% for the calculation of remaining percent activity. For symbols refer to Fig. 2.

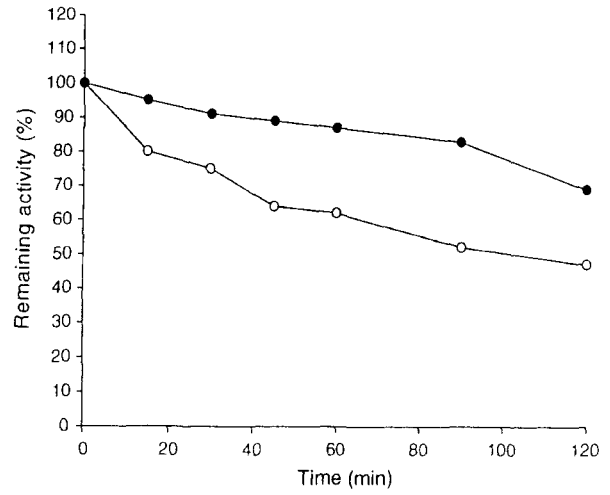

Fig. 5. Effect of 4.0 M urea on soluble and DEAE cellulose adsorbed BGP. Soluble and immobilized BGP (1.25 units) were incubated in 4.0 M urea in 50 mM sodium acetate buffer, pH 5.6. Enzyme activity was determined at different time intervals under conditions mentioned in the text. For calculating the percent activity untreated samples were considered as 100%. For symbols refer to Fig. 2.

3.3.5. *Protease mediated inactivation of soluble and DEAE cellulose bound BGP preparations*

Fig. 6 shows the stability of soluble and immobilized BGP in the presence of increasing concentration (0.25–2.5 mg) of trypsin/ml of incubation volume. Soluble BGP was rapidly inactivated in the presence of increasing concentrations of trypsin and retained 36% of the initial activity after 1 h incubation with 2.5 mg trypsin/ml at 37 °C while the immobilized BGP was remarkably more stable against proteolysis mediated by trypsin. However, the immobilized BGP showed over 55% of the original enzyme activity under similar treatment conditions.

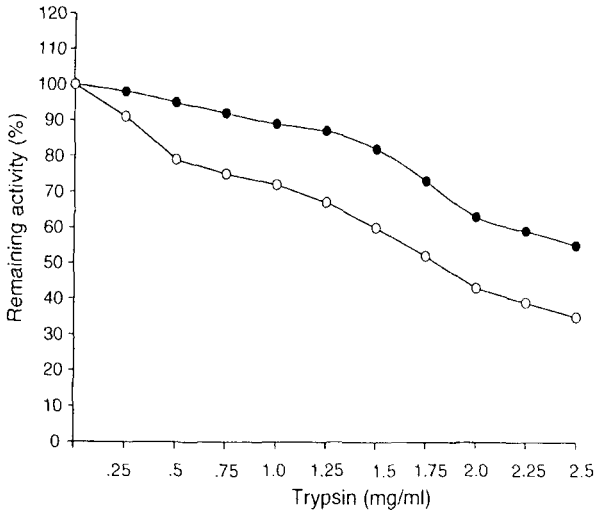

Fig. 6. Effect of trypsin concentration on the activity of soluble and DEAE cellulose adsorbed BGP. Soluble and DEAE cellulose adsorbed BGP (1.25 units) were independently incubated with increasing concentration of trypsin (0.25–2.5 mg) in a total volume of 1.0 ml of 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Activity of enzyme was assayed according to the procedure described in text. For symbols refer to Fig. 2.

3.3.6. *Effect of detergents on the activity of soluble and immobilized BGP*

In this study, three different detergents have been selected for comparative stability of soluble and immobilized BGP. Surf Excel and Rin powder are very commonly used detergent in every household and laundry. Unused detergents are normally present in the wastewater coming out of municipal waste. Somewhere such wastewater can mix with the effluents released by the industries. In order to make immobilized enzyme preparation more efficient for wastewater treatment, we have investigated the effect of Triton X 100, Surf Excel and Rin powder on the activity of soluble and immobilized BGP. Soluble BGP was more sensitive to the Surf Excel exposure and lost nearly 67% enzyme activity after 1 h incubation with 0.5% (w/v) detergent. Moreover, the immobilized BGP was markedly more resistant to inactivation induced by Surf Excel and retained over 70% of the initial activity (Table 2).

Table 2 further demonstrates the effect of increasing concentration of (0.1–1.0%) Rin powder on the activity of soluble and immobilized BGP. The soluble enzyme retained a marginal activity of 58% after 1 h exposure to 0.5% Rin powder. However, the immobilized BGP exhibited 85% of the original activity under similar exposure conditions.

Moreover, the immobilized BGP preparation was more resistant to denaturation induced by Triton X 100, this preparation retained 60% of the initial activity even in the presence of 5.0% (v/v) Triton X 100 whereas the soluble BGP retained only 38% of the original activity under similar exposure (Fig. 7).

Table 2
Effect of detergents on the activity of soluble and DEAE cellulose adsorbed BGP

Detergent concentration (%, w/v)	Percent remaining BGP activity			
	Surf Excel		Rin powder	
	Soluble	Immobilized	Soluble	Immobilized
0.1	105	111	99	147
0.2	94	103	98	142
0.3	69	81	93	137
0.4	57	77	83	127
0.5	33	70	58	85
0.6	22	58	25	60
0.7	21	52	20	49
0.8	14	46	12	32
0.9	11	26	8	25
1.0	7	22	6	21

Soluble and immobilized BGP preparations (1.25 units) were incubated with varying concentration of Surf Excel and Rin powder (0.1–1.0%, w/v) in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated detergent concentrations and other assay conditions were same as mentioned in the text. The activity of soluble and immobilized BGP in assay buffer without any detergent was taken as control (100%), for the calculation of percent activity. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

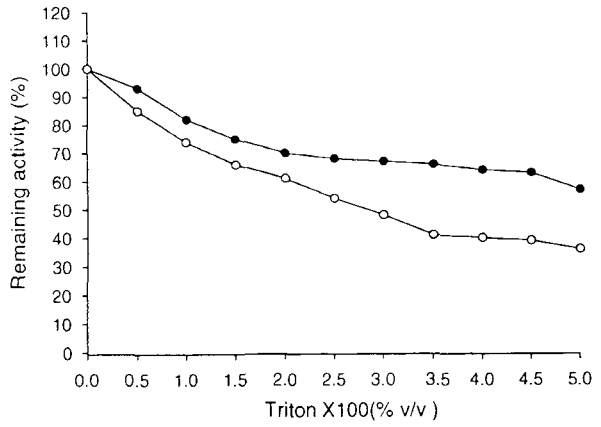

Fig. 7. Effect of Triton X 100 on soluble and DEAE cellulose adsorbed BGP. Soluble and immobilized BGP (1.25 units) were incubated with (0.5–5.0%, v/v) Triton X 100 prepared in 50 mM sodium acetate buffer, pH 5.6 at 37 °C for 1 h. Enzyme activity was determined after incubation period as described in the text. The activity of soluble and immobilized BGP in assay buffer without any detergent was taken as control (100%) for the calculation of percent activity. For symbols please refer to Fig. 2.

3.3.7. Effect of water-miscible organic solvents on the activity of soluble and immobilized BGP

Industrial effluents and wastewaters sometimes also contain organic solvents together with other aromatic pollutants and such compounds can affect the activity of enzymes during wastewater treatment. However, it is necessary to investigate the role of water-miscible organic solvents on the activity of immobilized enzymes. The exposure of soluble enzyme with varying concentrations of DMSO (10–60%, v/v) resulted in the loss of greater fraction of enzyme activity while the immobilized enzyme was quite resistant to inactivation induced by DMSO. Exposure of immobilized enzyme preparation with 50% (v/v) DMSO for 1 h retained 63% of its activity, although the soluble enzyme lost nearly 59% of its original activity under similar treatment (Table 3). The incubation of soluble and immobilized BGP with increasing concentrations of dioxane resulted in a continuous loss of enzyme activity. However, the immobilized enzyme was more resistant to inactivation mediated by dioxane. The treatment of soluble BGP with 60% (v/v) dioxane for 1 h resulted in a loss of 84% of the initial activity while the DEAE cellulose bound BGP exhibited stabilization against similar treatment and retained more than 35% of its original activity (Table 3). Table 3 summarizes the treatment of soluble and immobilized BGP with increasing concentration of *n*-propanol. The incubation of soluble enzyme with 60% (v/v) concentration of *n*-propanol resulted in decreasing more than 50% activity while immobilized enzyme showed retention of 70% of the initial activity.

4. Discussion

Several methods have been used for the immobilization/stabilization of enzymes but very few can meet the requirement of enzyme immobilization directly from the crude homogenate [11]. In this manuscript, an effort has been made to immobilize the peroxidases directly from the ammonium sulphate fractionated proteins of bitter gourd on DEAE cellulose. It is well documented that anion exchangers can be used for the purification of large number of proteins [15]. BGP was immobilized in very high yield on DEAE cellulose and it bound 590 units of BGP/g of the ion exchanger. The preparation thus obtained was highly active and exhibited very high effectiveness factor 'η' as 0.95 (Table 1). Effectiveness factor of an immobilized enzyme is a measure of internal diffusion and reflects the efficiency of the immobilization procedure [16]. In this case, the yield of immobilization was quite superior over other methods used for the immobilization of peroxidases [17–19]. DEAE cellulose adsorbed BGP was tightly retained as it has no significant detachment even in the presence of 0.5 M NaCl (Fig. 1). BGP bound to DEAE cellulose support exhibited very high stabilization against pH, heat and urea denaturation (Figs. 2–5). Several earlier investigators have also reported the use of DEAE cellulose support for high yield and stable immobilization of enzymes and proteins [20,21]. The immobilized BGP preparation exhibited broadening in pH–activity profiles (Fig. 2). We have earlier

Table 3
Effect of water-miscible organic solvents on the activity of soluble and immobilized BGP

Organic solvent (%, v/v)	Percent remaining BGP activity					
	Dioxane		DMSO		*n*-Propanol	
	Soluble	Immobilized	Soluble	Immobilized	Soluble	Immobilized
10	80	92	86	100	88	100
20	53	71	80	98	77	95
30	32	48	75	95	74	92
40	21	40	62	74	68	88
50	17	38	41	63	62	77
60	16	35	35	60	48	70

Soluble and immobilized BGP (1.25 units) preparations were incubated with increasing concentration of dioxane/DMSO/*n*-propanol (0–60%, v/v) in 50 mM sodium phosphate buffer, pH 5.6 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated organic solvent concentrations and other assay conditions were same as mentioned in the text. The activity of soluble and immobilized BGP in assay buffer without any water-miscible organic solvent were taken as control (100%) for the calculation of percent activity. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

shown that the Con A-Sephadex bound BGP had similar type of broadening in pH–activity profiles [6]. DEAE cellulose bound BGP was remarkably stable against proteolysis mediated by trypsin (Fig. 6). It has been earlier reported that Con A-Sephadex bound BGP was quite resistant to proteolysis induced by trypsin [6].

Wastewater coming out from various elimination sites contains several types of denaturants including detergents, which can strongly denature the enzymes used for the treatment of polluted wastewater. In order to use such enzymes for the removal of aromatic pollutants from wastewater it becomes necessary to monitor the stability of enzymes in presence of denaturants. The effect of detergents on the enzyme activity of immobilized BGP must be investigated prior to its application in the treatment of wastewater contaminated with hazardous organic compounds. Our observations suggested that DEAE cellulose bound BGP preparation was markedly more stable against the exposure caused by very high concentration of several detergents (Table 2; Fig. 7). DEAE cellulose bound enzyme could work more efficiently on industrial effluents containing compounds like soap and detergents. DEAE cellulose bound BGP was quite resistant against denaturation induced by detergents, such as Triton X 100 (Fig. 7), Rin powder and Surf Excel (Table 2). Lower concentrations of detergents enhanced the activity of immobilized BGP. These observations indicated the presence of lower concentrations of detergents is not harmful to the enzyme native conformation. The enhancement of enzyme activity by lower concentrations of detergents and stabilization of bioaffinity bound BGP against high concentrations of such type of detergents had already been reported by some earlier workers [6].

Organic solvents are also very common pollutants together with aromatic compounds and their presence can affect the structure of enzymes. Enzymes employed for the treatment of wastewater containing pollutants would be affected by the presence of such solvents. Due to presence of organic solvents in wastewater it necessitates the investigation of the stability of enzymes against inactivation induced by the exposure of such organic solvents. The DEAE cellulose bound BGP was markedly more stable when it exposed to dioxane, DMSO and propanol (Table 3). There have been reports that immobilization of enzymes by multipoint attachment protects them from denaturation induced by organic solvents in cosolvent mixtures [22,23]. These workers have described that potato polyphenol oxidase adsorbed on chitin behaved differently compared to soluble enzyme in aqueous-organic cosolvent mixtures. Moreover, they have evaluated that enzymes; polyphenol oxidase, peroxidase, trypsin and acid phosphatase showed stimulation of enzyme activity within a specific concentration range of water-miscible organic solvent present in the medium [24]. Enzyme immobilized by adsorption on Eudragit S-100, chitin and chitosan exhibited enhanced activity in organic cosolvent mixtures when the concentration of the organic solvent is around 10–20% (v/v) [9,10]. More recently, in our laboratory it has been shown that enzymes immobilized on protein supports were also quite resistant to denaturation induced by various water-miscible organic solvents [25,26].

DEAE cellulose bound BGP preparation has pronounced stability against pH, heat, urea, proteolysis, detergents and water-miscible organic solvents. Several earlier investigators have described that the immobilization of enzymes on DEAE cellulose support resulted in the stabilization of enzymes against various forms of denaturation [13,15,20]. Protease resistance is an additional attribute to the adsorption of BGP on an anion exchanger. It is expected that DEAE cellulose bound BGP preparation have a great future in the treatment of organic pollutants present in industrial effluents. DEAE cellulose adsorbed enzyme has only non-covalent forces between the support and enzyme molecules and sometimes it leads to the desorption of enzyme from the support. Adsorbed enzyme could be cross-linked by using bifunctional or multifunctional reagents in order to prevent the dissociation/desorption of enzyme from the ion exchanger [13,27].

5. Conclusion

Adsorption procedures are significantly useful in the immobilization of enzymes directly from the crude homogenate and thus avoiding the high cost of enzyme purification. The treatment of organic pollutants present in industrial effluents based on peroxidases has attracted considerable interest since the past two decades. However, practical applications of large-scale enzymatic removal have always been limited due to high cost and lower operational stability of peroxidases. Moreover, this procedure emphasized the immobilization of BGP directly from the crude homogenate or ammonium sulphate precipitated proteins. It has further reduced the cost of immobilized enzyme preparation. BGP adsorbed on DEAE cellulose support showed very high yield of immobilization and markedly high stabilization against several types of denaturants. In near future, the enzyme reactors containing such inexpensive immobilized enzyme preparations could be exploited for the treatment of wastewater containing toxic and hazardous compounds.

Acknowledgements

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Bioaffinity-based an inexpensive and high yield procedure for the immobilization of turnip (*Brassica rapa*) peroxidase

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Abstract

This study demonstrates the immobilization of carbohydrate containing turnip peroxidase on an inexpensive bioaffinity adsorbent, Concanavalin A-cellulose support. The bioaffinity support was prepared simply by incubating cellulose powder with jack bean extract at 4 °C. Cellulose powder adsorbed 30 mg concanavalin A/g of the matrix. Concanavalin A adsorbed cellulose has been employed for the simultaneous purification and immobilization of glycoenzymes directly from ammonium sulphate fractionated proteins of turnip. The obtained bioaffinity support was quite effective in high yield immobilization of peroxidase from turnip and it retained 672 U/g. Turnip peroxidase immobilized on concanavalin A-cellulose support retained 80% of the initial activity. Immobilized turnip peroxidase preparation was quite resistant against the denaturation mediated by pH, heat, urea, guanidinium-HCl, Surf Excel, cetyltrimethylammonium bromide and water-miscible organic solvents; dimethyl formamide, dioxane and *n*-propanol. Low concentration of detergents like Surf Excel and cetyltrimethylammonium bromide enhanced the activity of soluble and immobilized turnip peroxidase.

Keywords: Bioaffinity support; Concanavalin A; Cellulose; Immobilization; Peroxidase; Purification; Stabilization; Turnip roots

1. Introduction

Peroxidase (E.C. 1.11.1.7) are ubiquitous heme-proteins, which utilize hydrogen peroxide to catalyze the oxidation of a wide spectrum of organic and inorganic substrates (Duran et al., 2002; Duarte-Vazquez et al., 2003). Plant peroxidases are receiving increasing attention due to their extensive potential applications in clinical, biochemical, biotechnological, industrial and in the synthesis of useful compounds (Ryu et al., 1993; Lobarzewsky and Ginalska, 1995; Kim and Moon, 2005; Duran and Esposito, 2000). These enzymes could also be exploited for the detoxification and remediation of various aromatic pollutants such as phenols, aromatic amines, 2,4,6-trinitrotoluene and dyes, etc. present in wastewater/industrial effluents coming out from several industries such as textile, dyes, printing, paper and pulp (Husain and Jan, 2000; Akhtar et al., 2005a,b; Lee et al., 2003; McEldon and Dordick, 1996). The use of soluble enzymes has some inherent limitations whereas their immobilized form has several advantages over the soluble enzymes such as enhanced stability, easier product recovery and purification, protection of enzymes against denaturants, proteolysis and reduced susceptibility to contamination.

Numerous efforts have been made to develop the procedures for the immobilization of peroxidase from various sources but most of the immobilized enzyme preparations either use commercially available enzyme or expensive supports which increased the cost of the processes (Husain and Jan, 2000; Akhtar et al., 2005a; Tischer and Kasche, 1999). However, such immobilized enzyme preparations could not be exploited for the treatment of large volume of effluents coming out of the industrial sites. Among the techniques used for the immobilization of enzymes, bioaffinity supports have attracted the attention of the enzymologists due to several merits over the other known classical methods. Researchers have shown remarkable interest in the immobilization of enzymes on bioaffinity supports due to ease of immobilization, lack of chemical modification and usually accompanying an enhancement in stability (Saleemuddin and

Abbreviations: CTAB, cetyltrimethylammonium bromide; TP, turnip peroxidase; S-TP, soluble turnip peroxidase; I-TP, immobilized turnip peroxidase; Con A, concanavalin A; DMF, dimethyl formamide; SDS, sodium dodecyl sulphate

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Husain, 1991; Akhtar et al., 2005c; Saleemuddin, 1999). Besides the mentioned advantages offered by the bioaffinity-based procedures, there is some additional benefit, such as proper orientation of enzyme on the support (Turkova, 1999; Mislovicova et al., 2000; Khan et al., 2005). These supports have been used for high yield and stable immobilization of glycoenzymes/enzymes. A large number of bioaffinity-based procedures have already been developed for the immobilization of enzymes directly from the crude homogenate or partially purified enzyme preparation (Saleemuddin and Husain, 1991; Akhtar et al., 2005c; Saleemuddin, 1999; Khan et al., 2005).

In this article an effort has been made to select an inexpensive and easily available source of peroxidase, turnip. The purpose of this study was to find a cheaper and easily available alternative for the commercially available enzymes and its immobilization and utilization at large scale. Con A-cellulose immobilized turnip peroxidase (TP) preparation was compared with its soluble counter-part for its stability against various physical and chemical parameters.

2. Materials and methods

2.1. Materials

Methyl α-D-mannopyranoside was the products of Sigma Chem. Co. (St. Louis, MO), USA. Jack bean meal was procured from the Loba Chem. Co., India. *o*-Dianisidine-HCl was obtained from the Center for Biochemical Technology, New Delhi, India. Cetyltrimethylammonium bromide, dioxane, dimethyl formamide and *n*-propanol were obtained from the SRL Chemicals, Mumbai, India. Cellulose powder (0.02–0.15 mm) was obtained from Centron Research Labs, Mumbai, India. Surf Excel was the product of Hindustan Lever Ltd., Mumbai, India. Turnip roots were purchased from the local vegetable market. Other chemicals and reagents employed were of analytical grade and were used without any further purification.

2.2. Ammonium sulphate fractionation of turnip proteins

Turnip root (200 g) was homogenized in 200 ml of 0.1 M sodium acetate buffer, pH 5.5. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000 × *g* on a Remi Cooling Centrifuge C-24. The clear solution thus obtained was subjected to salt fractionation by adding 20–80% (w/v) $(NH_4)_2SO_4$. The mixture was stirred overnight at 4 °C to obtain the maximum precipitate. The precipitate was collected by centrifugation at 10,000 × *g* on a Remi Cooling Centrifuge C-24. The obtained precipitate was re-dissolved in 0.1 M sodium acetate buffer, pH 5.5 and dialyzed against the assay buffer (Matto and Husain, 2006).

2.3. Preparation of bioaffinity support

Cellulose (5.0 g) was incubated and stirred with 100 ml of clear solution of jack bean extract prepared in 0.1 M sodium phosphate buffer, pH 6.2 overnight at 4 °C. The unbound proteins were removed by extensive washing with assay buffer (Akhtar et al., 2005c). The specific binding of Con A with cellulose was confirmed by eluting the bound lectin using 1.0 M methyl α-D-mannopyranoside.

2.4. Measurement of peroxidase activity

Peroxidase activity was determined from a change in the optical density ($A_{460\ nm}$) by measuring the initial rate of oxidation of 6.0 mM *o*-dianisidine-HCl in the presence of 18 mM hydrogen peroxide in 0.1 M sodium acetate buffer, pH 5.5 for 15 min at 37 °C.

The immobilized preparation was continuously agitated for the entire duration of assay. The assay was highly reproducible with immobilized preparations (Musthapa et al., 2004).

One unit of peroxidase activity (U) was defined as the amount of enzyme protein that catalyzes the oxidation of 1 (mol of *o*-dianisidine-HCl per min at 37 °C into colored product ($\varepsilon_m = 30.000\ M^{-1}\ L^{-1}$).

2.5. Immobilization of TP on Con A-cellulose support

TP (7240 U) were added to 5.0 g of Con A-cellulose support and stirred in sodium phosphate buffer, pH 6.2 at 4 °C overnight. The unbound TP was removed by extensive washing with the assay buffer (Akhtar et al., 2005c).

2.6. Effect of pH on soluble and immobilized TP

The activities of soluble and immobilized TP preparations (1.15 U) were measured in buffers of various pH values (3.0–10.0). The molarity of each buffer was 0.1 mol L^{-1}.

2.7. Effect of temperature on soluble and immobilized TP

The activities of soluble and immobilized TP preparations (1.15 U) were measured at various temperatures (20–80 °C) under standard assay conditions. The activity obtained at 30 °C was taken as 100% for the calculation of percent activity.

Soluble and immobilized TP preparations (1.15 U) were incubated at 60 °C in 0.1 M sodium acetate buffer, pH 5.5. Aliquots of each preparation were removed at each indicated time interval and activity was measured. The activity obtained without incubation at 60 °C was taken as control (100%) for the calculation of percent activity.

2.8. Effect of detergents on soluble and immobilized TP

Surf Excel (0.1–1.0%, w/v) and CTAB (0.2–2.0%, w/v) were used as final assay concentration to observe the effect of detergents on the activity of TP. Soluble and immobilized TP preparations (1.15 U) were incubated with the detergents in 0.1 M sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was determined at all the indicated detergent concentrations. The activity obtained without exposure to detergent was taken as 100% for the calculation of percent activity.

2.9. Effect of water-soluble organic solvents on soluble and immobilized TP

Soluble and immobilized TP preparations (1.15 U) were incubated with 10–60% (v/v) of water-miscible organic solvents; DMF/dioxane/*n*-propanol prepared in 0.1 M sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was determined at all the indicated organic solvent concentrations after the incubation period (Jan et al., 2001). Other assay conditions were the same as described in the text.

2.10. Determination of protein concentration

The protein concentration was determined according to the procedure described by Lowry et al. (1951). Bovine serum albumin was used as standard.

3. Results

3.1. Preparation of bioaffinity support and immobilization of TP

Cellulose adsorbed nearly 30 mg protein/g cellulose powder from jack bean extract. Con A-cellulose matrix was selected as a bioaffinity media for the direct immobilization of

Table 1
Immobilization of TP on Con A-cellulose support

Amount of enzyme loaded, X (U)	1448
Amount of enzyme activity in washes, Y (U)	608
Activity bound/g Con A-cellulose support (U)	
Theoretical ($X - Y = A$) (A)	840
Actual (B)	672
Effectiveness factor (η) (B/A)	0.8
Percentage of activity yield ($B/A \times 100$)	80%

Peroxidase activity was assayed according to the procedure described in Section 2.4. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

glycoenzymes from ammonium sulphate fractionated turnip proteins. It has already described that some of the isoenzymes of turnip peroxidases are glycosylated in nature (Duarte-Vazquez et al., 2003). In view of the glycoproteinic nature of turnip peroxidases, these enzymes could be directly immobilized on Con A-cellulose support from ammonium sulphate fractionated proteins or from the crude homogenate of turnip. The unbound proteins were removed by extensive washing with assay buffer. Con A-cellulose adsorbed 672 U of peroxidase per g of the matrix (Table 1).

The stability of soluble and Con A-cellulose bound TP preparation was monitored against various physical and chemical parameters because these parameters can influence the activity of the enzymes used for the treatment of organic pollutants present in the wastewater.

3.2. pH-activity profile

Fig. 1 shows the pH-activity profile of soluble and Con A-cellulose bound TP. Soluble and immobilized both TP preparations showed same pH-optima at pH 5.0. However,

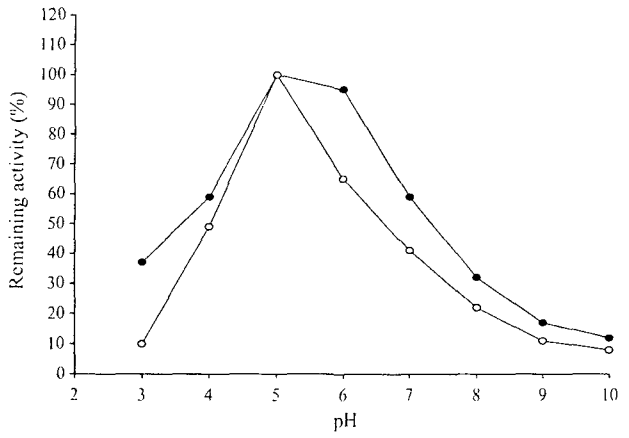

Fig. 1. pH-activity profiles of the soluble and immobilized TP. The appropriate amount of soluble and immobilized TP was taken for the preparation of pH-activity profile. The reaction mixture was incubated at 37 °C for 15 min in buffers of pH ranges from 3.0 to 10.0. The buffers used were 0.1 M glycine-HCl for the pH 3.0, sodium acetate for the pH 4.0, 5.0, sodium phosphate for the pH 6.0, 7.0, 8.0 and Tris–HCl for the pH 9.0 and 10.0. Activity expressed at pH 5.0 was considered as control (100%) for the calculation of percent activity. Symbols indicate, the soluble (○) and immobilized (●) TP.

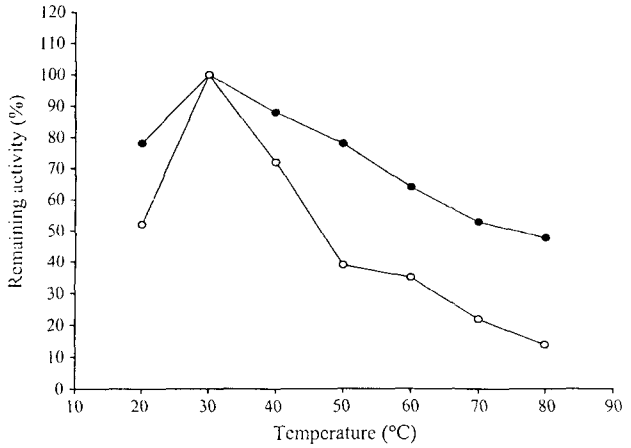

Fig. 2. Temperature-activity profiles of soluble and immobilized TP. The activity of appropriate amount of soluble and immobilized TP was monitored at various indicated temperatures. Activity expressed at 30 °C was taken as control (100%) for the calculation of percent activity. For symbols refer to the legend of Fig. 1.

the immobilized TP preparation exhibited higher percent of activity at alkaline side of pH-optima.

3.3. Effect of temperature

Bioaffinity bound TP preparation exhibited a marginal broadening in temperature-activity profile. Soluble and Con A-cellulose bound TP preparations showed same temperature-optima at 30 °C. Con A-cellulose adsorbed TP retained greater fractions of catalytic activity at higher temperatures as compared to the free enzyme (Fig. 2). Soluble TP retained a marginal activity of 22% at 70 °C whereas the immobilized enzymes exhibited more than half of the maximum activity.

Fig. 3 demonstrates the thermal denaturation of soluble and immobilized TP at 60 °C. Immobilized TP incubated at 60 °C

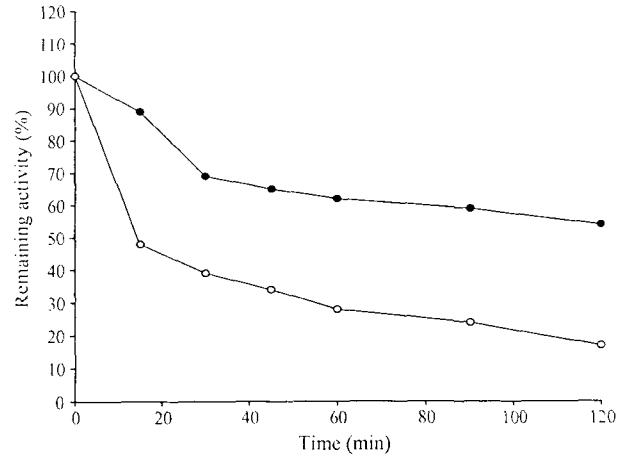

Fig. 3. Thermal denaturation of soluble and Con A-cellulose adsorbed TP. Soluble and immobilized TP preparations (1.15 U) were independently incubated at 60 °C for varying time intervals in 0.1 M sodium acetate buffer, pH 5.5. Aliquots of each preparation were taken out at indicated time intervals and enzyme activity was determined. Unincubated samples were taken as control (100%) for the calculation of remaining percent activity. For symbols refer to the legend of Fig. 1.

for 2 h retained 54% of the initial enzyme activity while the soluble enzyme lost nearly than 83% of the original activity under similar incubation conditions.

3.4. Effect of urea and guanidinium-HCl

Con A-cellulose bound TP was more resistant to inactivation induced by 4.0 M guanidinium-HCl compared to its soluble counterpart. Immobilized enzyme preparation retained more than half of the initial enzyme activity when exposed to 4.0 M guanidinium-HCl for 1 h while the soluble TP exhibited marginally 15% of the initial activity under similar incubation conditions (Table 2).

Table 2 further demonstrates the treatment of soluble and immobilized TP preparations with 4.0 M urea for various time periods. Immobilized TP was significantly more stable against the denaturation mediated by urea and this preparation retained over 85% of the enzyme activity after 2 h exposure with 4.0 M urea. However, the soluble enzyme was more sensitive to urea treatment and lost more than 65% of the initial TP activity after 2 h treatment with 4.0 M urea concentration (Table 2).

3.5. Effect of detergents

Wastewater coming out from various industrial sites contains several types of denaturants including detergents, which can strongly denature the enzymes used for the treatment of polluted wastewater. In order to use such enzymes for the treatment of aromatic pollutants from wastewater it becomes necessary to monitor the stability of enzymes in the presence of some detergents. In this study different detergents have been selected for comparative stability of soluble and immobilized TP. Surf Excel is a very common detergent used in household and laundry. Unused detergent is normally present in the wastewater coming out of municipal waste. This wastewater is mixed with the effluents released by the industries. In order to monitor the compatibility of the immobilized enzyme in such treatments, the effect of Surf Excel on the activity of immobilized TP should be checked. Soluble TP was more sensitive to the Surf Excel exposure and lost 90% of the initial enzyme activity after 1 h exposure with 1% (w/v) detergent. However, the immobilized TP was markedly more resistant to inactivation induced by Surf Excel and retained over 56% of the initial activity (Fig. 4). Both soluble and immobilized enzyme preparations were activated by low concentrations of Surf Excel, however, the immobilized TP achieved further activation at higher levels of detergent (Fig. 4). This experiment indicated that the presence of lower concentrations of Surf Excel is not harmful to the enzyme structure and enzymes could work more effectively on the industrial effluents containing compounds like soap and detergents. Lower concentrations of Triton X 100 and Tween 20 activated soluble and immobilized TP and in this case the extent of activation was much higher (data not shown). In order to investigate the effect of higher concentrations of detergents on the activity of TP, soluble and immobilized TP preparations were further incubated with 0.2–2.0% (w/v) CTAB, a cationic detergent for 1 h at 37 °C. Pre-incubation of soluble and immobilized enzyme preparations with 2% (w/v) CTAB for 1 h exhibited enhanced activity of 131% and 225% of the original enzyme activity, respectively (Fig. 5).

Table 2
Effect of urea and guanidinium-HCl on soluble and immobilized TP

Time of incubation (min)	Percent remaining TP activity			
	Urea (4.0 M)		Guanidinium-HCl (4.0 M)	
	S-TP	I-TP	S-TP	I-TP
15	93	100	31	70
30	92	100	28	64
45	90	100	19	56
60	84	95	15	52
90	64	93	14	32
120	34	86	13	27

Soluble and immobilized TP preparations (1.15 U) were independently incubated with 4.0 M urea/guanidinium-HCl prepared in 0.1 M sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was assayed at different time intervals under conditions mentioned in Section 2.4. The activity of soluble and immobilized TP in assay buffer without any denaturant was taken as control (100%) for the calculation of percent activity. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

3.6. Effect of water-miscible organic solvents

Wastewater coming out of industrial sites also contains several types of solvents along with other aromatic pollutants. In view of their presence in industrial effluents, it became more important to examine the effect of some water-miscible organic solvents on the activity of immobilized enzymes. The exposure of soluble enzyme with varying concentration of DMF (10–60%, v/v) resulted in the loss of greater fraction of enzyme

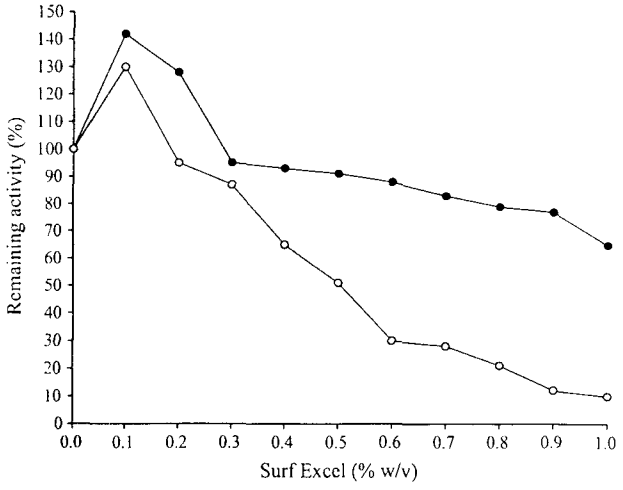

Fig. 4. Effect of Surf Excel on the activity of soluble and immobilized TP. Soluble and immobilized TP (1.15 U) were incubated with varying concentrations of Surf Excel (0.1–1.0%, w/v) in 0.1 M sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Enzyme activity was determined after incubation period as described in Section 2.4. The activity of soluble and immobilized TP in assay buffer without any concentration of Surf Excel was taken as control (100%) for the calculation of percent activity. For symbols please refer to the legend of Fig. 1.

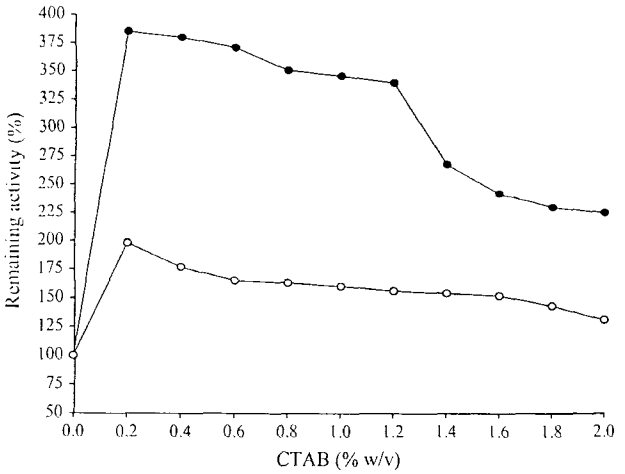

Fig. 5. Effect of CTAB on the activity of soluble and immobilized TP. Soluble and immobilized TP (1.15 U) were incubated with varying concentrations of CTAB (0.2–2.0%, w/v) in 0.1 M sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Enzyme activity was determined after incubation period as described in Section 2.4. The activity of soluble and immobilized TP in assay buffer without any CTAB was taken as control (100%) for the calculation of percent activity. For symbols please refer to the legend of Fig. 1.

activity while the immobilized enzyme was quite resistant to inactivation induced by DMF. Con A-cellulose bound TP preparation retained nearly 70% of the original activity after 1 h exposure with 60% (v/v) DMF whereas the soluble enzyme lost nearly 90% of the activity under similar treatment conditions (Table 3).

The exposure of soluble and Con A-cellulose adsorbed TP with (10–60%, v/v) dioxane showed a continuous decrease in peroxidase activity. Immobilized TP had retained greater fraction of catalytic activity at the exposure of various concentrations of dioxane (Table 3). The incubation of soluble and immobilized TP with increasing concentration of *n*-propanol resulted in a continuous loss of enzyme activity. Moreover, the immobilized enzyme preparation exhibited more resistant to inactivation induced by *n*-propanol. The treatment of soluble TP with 60% (v/v) *n*-propanol for 1 h resulted in a loss of 88% of the initial activity while the Con A-cellulose bound TP exhibited significantly higher stabilization against similar treatment and retained more than 50% of the initial activity (Table 3).

Table 3
Effect of organic solvents on soluble and immobilized TP

Organic solvent (%, v/v)	Percent remaining TP activity					
	DMF		Dioxane		*n*-Propanol	
	S-TP	I-TP	S-TP	I-TP	S-TP	I-TP
10	87	97	76	88	76	92
20	75	80	41	58	54	62
30	56	79	26	41	20	58
40	34	78	13	38	18	57
50	20	74	11	30	16	53
60	10	69	10	23	12	51

Soluble and immobilized TP preparations (1.15 U) were independently incubated with increasing concentration of DMF/dioxane/*n*-propanol (10–60%, v/v) in 0.1 M sodium acetate buffer, pH 5.5 at 37 °C for 1 h. Peroxidase activity was assayed at all the indicated organic solvent concentrations. The activity of soluble and immobilized TP in assay buffer without any organic solvent was taken as control (100%) for the calculation of percent activity. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

4. Discussion

Several methods have been described for the immobilization/stabilization of enzymes but very few of them meet the requirement of enzyme immobilization directly from the crude homogenate or partially purified enzyme preparations. Here a simple and an elegant approach have been applied to immobilize TP directly from ammonium sulphate fractionated proteins of turnip roots on Con A-cellulose. It is now well recognized that polysaccharides could be used for bioaffinity-based purification of Con A from the jack bean extract (Saleemuddin and Husain, 1991). However, such property has been exploited for the preparation of bioaffinity media for the immobilization of enzymes from crude preparations. TP was immobilized in very high yield on Con A-cellulose support and it retained 672 U of TP/g of the adsorbent. The immobilization yield was quite superior over other methods used for the immobilization of peroxidases (Akhtar et al., 2005c; Husain et al., 1992). Con A-cellulose bound TP exhibited very high stabilization against the inactivation induced by pH, heat, urea and guanidinium-HCl denaturation (Figs. 1–3 and Table 2). Several earlier investigators have also reported about the use of Con A support for high yield and stable immobilization of glycoenzymes (Saleemuddin and Husain, 1991; Saleemuddin, 1999).

Lower concentrations of various detergents enhanced activity of soluble and immobilized TP. These experiments indicated that the presence of lower concentrations of detergents is not harmful to the enzyme function. Such enzymes can work more efficiently on industrial effluents containing compounds, i.e. detergents. Con A-cellulose adsorbed TP was quite resistant against denaturation induced by detergents such as Triton X 100, Tween 20 (data not given), Surf Excel and CTAB (Figs. 4 and 5). Numerous detergents normally flow in the municipal wastewater and these could affect the activity of enzymes. Our observations suggested that Con A-cellulose bound TP preparation was remarkably more stable against the exposure caused by high concentration of several detergents. Potential applications of this enzyme could be used for the treatment of wastewater containing hazardous aromatic pollutants.

Organic solvents are also very common pollutants along with aromatic compounds and their presence can influence the structure of enzymes. Enzymes exploited for the treatment of wastewater containing aromatic pollutants would be affected by the presence of water-miscible organic solvents. The Con A-cellulose bound TP was remarkably more resistant against the inactivation mediated by DMF, dioxane and *n*-propanol (Table 3). It has been already reported that immobilization of enzymes by multipoint attachment protects them from

denaturation mediated by water-miscible organic solvents (Mozhaev et al., 1990; Fernandez-Lafuente et al., 1995; Batra and Gupta, 1994). Akhtar et al. (2005c) have demonstrated that bitter gourd peroxidase immobilized on Con A-Sephadex support behaved differently compared to soluble enzyme in aqueous–organic co-solvent mixtures. More recently in our laboratory it has been shown that enzymes immobilized on protein supports were also quite resistant to denaturation induced by various water-miscible organic solvents (Matto and Husain, 2006; Jan et al., 2001; Jan and Husain, 2004). Fernandes et al. (2003) improved HRP organic solvent tolerance by immobilization, though to at a lesser extent since at 20% organic solvent concentration HRP-PANIG$_1$ retained between 60% (acetone) and 19% (acetonitrile) activity. In a more recent study Magri et al. (2005) have also reported that immobilized soybean seed coat peroxidase shows full activity over the organic solvent concentration range (5–70%, v/v) assayed whereas the free enzyme was almost inactive in 50% (v/v) of the solvents assayed. Enzymatic catalysis in organic solvents is possible if the active-site structure is not substantially disturbed by the organic solvent and if enough water is present (Ryu and Dordick, 1992); it seems that in the case of Con A-cellulose bound TP both conditions are met.

Con A-cellulose bound TP preparation has pronounced stability against pH, heat, urea, guanidinium-HCl, detergents and water-miscible organic solvents. Earlier reports described that the immobilization of glycoenzymes on Con A support resulted in the stabilization of enzymes against several types of denaturation (Saleemuddin and Husain, 1991; Mislovicova et al., 2000). It suggested that Con A-cellulose bound TP preparation has great potential in the treatment of organic pollutants present in industrial effluents. Con A-cellulose adsorbed enzyme has only non-covalent forces between the support and enzyme molecules and sometimes it led to the desorption of enzyme or Con A or both from the support. Cross-linking of bioaffinity adsorbed enzyme could be done by using bifunctional or polyfunctional reagents to prevent the dissociation/desorption of enzyme or Con A-glycoenzyme complex from the cellulose support (Jan et al., 2006).

5. Conclusion

The procedure for the immobilization of proteins, developed in this study exhibited its own merits due to use of crude jack bean extract, the source of lectin and ammonium sulphate fractionated turnip proteins, source of enzyme. Moreover, this procedure emphasized the immobilization of TP directly from the crude homogenate or ammonium sulphate fractionated proteins on bioaffinity support. TP adsorbed on Con A support showed very high yield of immobilization and markedly high stabilization against several forms of denaturants. In near future, the reactors containing such types of inexpensive immobilized enzyme preparations could be exploited for the treatment of wastewater containing toxic and hazardous compounds.

Acknowledgements

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Decolorization and degradation of acid dyes mediated by salt fractionated turnip (*Brassica rapa*) peroxidases

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Abstract

Peroxidases from turnip roots (524 U g^{-1} of vegetable) were highly effective in decolorizing acid dyes having wide spectrum chemical groups. Dye solutions, containing 40–170 mg dye L^{-1}, were treated by turnip peroxidases (TP) (specific activity of 122.0 U mg^{-1} proteins). These enzymes were able to decolorize most of the acid dyes in the presence of 2.0 mM 1-hydroxybenzotriazole (HOBT). Increasing concentration of enzyme and time in the absence of HOBT did not influence dye decolorization. The rate of decolorization was significantly enhanced when HOBT was added to the decolorizing solutions. The decolorization of all the used dyes was maximum at pH 5.0 and 40°C. Complex mixtures of dyes were significantly decolorized when treated with enzyme in the presence of HOBT (2.0 mM). Phytotoxicity test based on *Allium cepa* root growth inhibition has shown that majority of the TP-treated dye product were not more toxic than their parent dye. Kinetic parameters of the TP with various dyes showed that this enzyme has highest affinity for Acid Yellow 42. This study demonstrates that the peroxidase/mediator system was an effective biocatalyst for the treatment of industrial effluents from textile, dye manufacturing, dyeing and printing industries or complex mixtures of dyes.

Keywords: *Turnip, peroxidases, 1-hydroxybenzotriazole, dye, degradation*

Abbreviations: TP, Turnip peroxidases; HOBT, 1-hydroxybenzotriazole; HRP, horseradish peroxidase; BGP, bitter gourd peroxidase

Introduction

Pollution of communal water bodies by waste dyestuff released from textile plants and dye houses represents a major environmental concern. There is currently a considerable

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environmental interest for color removal from a wide range of wastewater. In case of the textile manufacturing industry, up to 50% of the dyes are lost after dyeing process which are disposed out in the effluents [1,2]. Most of the dyes used in textile industry are classified as cationic, anionic or non-ionic type. Anionic dyes are the direct, acid and reactive dyes [3]. Acid dyes comprise of one of the classes of water soluble synthetic dyes with greatest variety of colors and structures. Although presently a wide range of physical and chemical methods is available to decolorize dye-contaminated effluents [4], but these methods are outdated due to some unresolved problems. However alternative procedures based on biotechnological principles are attracting increasing interest [2,5].

Dyes are removed by a wide variety of aerobic or anaerobic organisms, which are preferably employed as mixed cultures because of their relative robustness and versatility against xenobiotic compounds [1,6,7]. Recently, a pilot plant containing a combination of anaerobic/aerobic organisms has been developed for the treatment of colored textile effluents [5]. One major advantage of such systems is the complete mineralization often achieved due to synergistic action of different organisms [8].

However, the biological procedures have their own limitations, such as the non-biodegradability of the xenobiotic compounds due to lack of requisite enzymes in the biological treatment plant [2,7]. Often the environment of the microorganisms is not optimal for rapid degradation of pollutants [1,2]. There is a need to find alternative procedures for their treatment that are effective in removing dyes from large volume of effluents and are low in cost [9].

Recently, enzymatic approach has attracted much interest in the removal of phenolic pollutants from aqueous solutions as an alternative strategy to the conventional chemical as well as microbial treatments that pose some serious limitations [10–12]. Oxidoreductive enzymes; peroxidases and polyphenol oxidases are participating in the degradation/removal of aromatic pollutants from various contaminated sites [13]. These enzymes can act on a broad range of substrates that can also catalyze the decolorization and decontamination of organic pollutants, even if they are present in a very low concentration at the contaminated site. In view of the potential of the enzymes in treating the phenolic compounds several microbial and plant oxidoreductases have been employed for the treatment of dyes, but none of them has been exploited at large scale due to low enzymatic activity in biological materials and high cost of enzyme purification [13–16].

In this study, we have investigated the role of partially purified turnip peroxidases (TP) for the degradation/decolorization of acid dyes, having a wide spectrum of chemical groups, currently being used by the textile industries. The majority of the tested dyes were recalcitrant to decolorization/degradation by TP. However, the addition of 2.0 mM 1-hydroxybenzotriazole (HOBT), a redox mediator, to the reaction mixture enhanced the rate of decolorization of these dyes several folds. The mixtures of dyes were also successfully decolorized by TP. Kinetic parameters of the TP with various dyes were also determined in order to examine the affinity of the enzyme for dyes.

Materials and methods

Materials

Acid dyes were a gift from Atul Chemicals, Ltd. India. Ammonium sulfate and HOBT were purchased from SRL Chemicals, Mumbai, India. *o*-Dianisidine-HCl was obtained from the IGIB, New Delhi, India. Turnip used in the study was purchased from a local

vegetable market. The chemicals and other reagents employed were of analytical grade and were used without any further purification.

Ammonium sulfate fractionation of turnip proteins

Turnip (50 g) was homogenized in 100 mL of 100 mM sodium acetate buffer, pH 5.5. Homogenate was filtered through four layers of cheesecloth. The filtrate was then centrifuged at 10,000 *g* on a Remi C-24 Cooling Centrifuge. The clear supernatant thus obtained was subjected to salt fractionation by adding 20–80% (w/v) $(NH_4)_2SO_4$. The mixture was stirred overnight at 4°C to obtain maximum precipitate. The precipitate was collected by centrifugation at 10,000 *g* on a Remi C-24 Cooling Centrifuge. The obtained precipitate was re-dissolved in an appropriate volume of 100 mM sodium acetate buffer, pH 5.5 and dialyzed against the assay buffer [17].

Treatment of dyes with increasing concentration of TP

The dyes (40–170 mg L^{-1}) were prepared in 100 mM sodium acetate buffer, pH 5.0. Each dye was incubated with increasing concentration of TP "0.117–0.352 U mL^{-1} of reaction volume" in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H_2O_2 for 1 h at 37°C. HOBT (2.0 mM) was used as a redox mediator for the selected experiments. Dye decolorization by TP was monitored at their respective wavelength maxima in the presence and absence of 2.0 mM HOBT. The percent decolorization was calculated by taking untreated dye solution as control (100%).

Treatment of dyes with fixed concentration of TP for varying times

Each dye was incubated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 37°C in the presence of 0.75 mM H_2O_2 for varying time intervals; 15 min to 2 h. Decolorization was also performed in the presence of 2.0 mM HOBT under other similar experimental conditions. The decrease in absorbance was monitored at predetermined intervals at the respective λ_{max} of each dye. The percent decolorization was calculated by taking untreated dye solution as control (100%).

Allium cepa *test for TP-treated dyes*

The *Allium cepa* bioassay for the treated dye sample was carried out according to the method of Fiskesjo [18]. For this test small onions of equal size are taken and by using a sharp knife the yellowish brown outer scales and brownish bottom plates are removed carefully leaving the ring primordial intact. Boiling tubes filled with treated dye sample consisting of 0.235 U mL^{-1} of TP in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H_2O_2 and 2.0 mM of HOBT are used to remain in contact with onion bulbs. Aqua guard water is used as control in all experiments, and the experiments are performed in dark conditions.

One onion is placed at the top of each tube with root primordial downward touching the liquid. After a gap of 12 h the same samples are added in the tube to fill up to the top and care is taken that there should be no gap between onion bulb and sample present in the test tube.

The treatment is continued for 7 days. After completion of the time of treatment onions are taken out, and for each sample root length are measured. Inhibitions in the growth of *A. cepa* roots are considered as an index for the degree of toxicity [18].

Effect of pH on the decolorization of dyes by TP

In this experiment the dyes were prepared in the buffers of different pH values (3.0–10.0). Each dye was treated with TP (0.235 U mL^{-1}) in the buffers of various pH values in the presence of 0.75 mM H_2O_2 for 1 h at 37°C. HOBT (2.0 mM) was used as a redox mediator for the dye decolorization. Dye decolorization by TP was monitored at the respective wavelength maxima of each dye. The percent decolorization was calculated by taking each untreated dye in specific buffer as control (100%).

Effect of temperature on the decolorization of dyes by TP

Each dye was incubated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 and 0.75 mM H_2O_2 at 30–80°C for 1 h. HOBT (2.0 mM) was used as a redox mediator for the dye decolorization. Decrease in color of dyes after treatment with TP was monitored at specific wavelength maxima of each dye. The percent decolorization was calculated by taking untreated dye solution incubated at each temperature as control (100%).

Decolorization of mixture of dyes by TP

Dye mixtures were prepared by mixing different dyes in equal proportions in terms of absorbance. The mixtures of dyes were treated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 in the presence of 0.75 mM H_2O_2 and 2 mM of HOBT for 1 h at 37°C. Decrease in absorbance in each TP-treated dye mixture was monitored at specific wavelength maxima of the mixture. The percent decolorization was calculated by taking untreated dye mixture as control (100%).

Determination of K_m and V_{max} of the TP with respect to tested dyes

The initial rates of enzymatic dye degradation were measured at various concentrations of the dye. In this experiment, the solutions having different dye concentrations ranging from 15–200 mg L^{-1} were treated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 and 0.75 mM H_2O_2 at 37°C for 1 h. HOBT (2.0 mM) was used as a redox mediator for the dye decolorization.

UV–Visible spectral analysis

Procedure for the dye decolorization was followed by UV–Vis spectral analysis. Spectra for the control and TP-treated dye samples were taken on Cintra 10*e* UV–Vis spectrophotometer.

Assay of TP activity

Peroxidase activity was measured from the change in the optical density (A_{460} nm) at 37°C by measuring the initial rate of oxidation of *o*-dianisidine-HCl by H_2O_2 using the two substrates in saturating concentrations [19].

One unit of peroxidase activity was defined as the amount of enzyme protein that catalyzes the oxidation of *o*-dianisidine-HCl in the presence of H_2O_2 into 1.0 μmol of chromophoric complex ($\varepsilon_m = 30{,}000\ M^{-1}\ cm^{-1}$) per min at 37°C.

Determination of protein concentration

The protein concentration was determined by the procedure described by Lowry et al. [20]. Bovine serum albumin was used as standard.

Results and discussion

Turnip root is a good source of peroxidase, and because of their kinetic and biochemical properties have a high potential as an economic alternative to the other commercially available plant and microbial peroxidases.

TP catalyzed polymerization and precipitation of various phenolic compounds is well-documented in the literature [21,22]. However, the decolorization and degradation of dyes by TP has not yet been reported. Therefore, for the first time, we have investigated the role of TP in the decolorization of industrially important acid dyes used in textile industry. Ammonium sulfate precipitated proteins from turnip root were taken for the treatment of a number of acid dyes. Partially purified preparation of TP was obtained by adding 20–80% ammonium sulfate, and this preparation exhibited a specific activity of 122.0 U mg^{-1} proteins. The experiments were designed to investigate the dye decolorization in the presence of H_2O_2 and partially purified TP. The dye solutions were found to be stable upon exposure to H_2O_2 or to the enzyme alone.

Treatment of dyes by varying TP concentration

Table I summarizes the decolorization of five acid textile dyes by using increasing concentration of TP (0.117–0.352 U mL^{-1} of reaction volume) for 1 h at 37°C. An increase in the enzyme activity has resulted in a continuous enhancement in the rate of dye decolorization. Acid Blue 92 was decolorized 61% with 0.352 U of TP mL^{-1} of reaction mixture whereas Acid Red 97, Acid Yellow 42, Acid Black 1 and Acid Black 210 were recalcitrant to the TP action (Table I).

Table I. Effect of TP concentration on the decolorization of acid dyes.

		Decolorization (%)					
		Enzyme unit mL^{-1} of reaction volume					
		0.117		0.235		0.352	
Name of the dye	λ_{max} (nm)	(−)	(+)	(−)	(+)	(−)	(+)
Acid Blue 92	564	13	70	21	100	61	100
Acid Red 97	497	0	27	0	86	0	92
Acid Yellow 42	408	0	74	0	91	0	91
Acid Black 1	619	0	26	0	87	0	87
Acid Black 210	456	0	24	0	62	0	65

Each dye was treated with increasing concentrations of TP (0.117–0.352 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 37°C for 1 h. HOBT (2.0 mM) was used as a redox-mediator for the selected experiments. In this table (−) and (+) signs indicate the decolorization of dyes in the absence and presence of HOBT, respectively. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

Table II. Treatment of acid dyes with fixed concentration of TP for varying times.

	Decolorization (%)											
	Time (min)											
	15		30		45		60		90		120	
Name of the dye	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)
Acid Blue 92	7	68	15	76	17	98	21	100	22	100	25	100
Acid Red 97	0	72	0	80	0	84	0	86	0	91	0	92
Acid Yellow 42	0	91	0	91	0	91	0	91	0	91	0	91
Acid Black 1	0	73	0	75	0	77	0	87	0	87	0	87
Acid Black 210	0	37	0	41	0	62	0	62	0	63	0	65

Each dye was treated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 37°C for varying time periods. HOBT (2.0 mM) was used as a redox-mediator for the selected experiments. In this table (−) and (+) signs indicate the decolorization of dyes in the absence and presence of HOBT, respectively. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

Treatment of dyes for varying times with fixed concentration of TP

Acid dyes were incubated with 0.235 U of TP mL^{-1} of reaction volume for increasing time period. Out of five, only Acid Blue 92 were decolorized on treatment with TP for 2 h at 37°C. Although more color disappeared when dye was incubated for longer durations, the rate of decolorization was slow (Table II). Rest of the four dyes; Acid Red 97, Acid Yellow 42, Acid Black 1 and Acid Black 210 were fully recalcitrant to decolorization by TP even after a longer period of incubation under similar conditions.

Treatment of dyes in the presence of redox-mediator

Five acid dyes used in this study were treated with TP in the presence of 2.0 mM HOBT and 0.75 mM H_2O_2 at 37°C. Presence of HOBT drastically enhanced the rate of decolorization of recalcitrant dyes (Table I). Acid Red 97, Acid Yellow 42, Acid Black 1 and Acid Black 210 were recalcitrant to decolorization in the absence of HOBT. However, these dyes were decolorized up to 92, 91, 87 and 65%, respectively by the action of 0.352 U mL^{-1} of reaction volume in the presence of 2.0 mM HOBT at 37°C for 1 h.

Role of HOBT in the decolorization of recalcitrant dyes is further noticeable in Table II. The decolorization of dyes with 0.235 U of TP mL^{-1} of reaction volume at 37°C for 1 h in the presence of 2.0 mM HOBT was 86% for Acid Red 97, 91% for Acid Yellow 42, 87% for Acid Black 1 and 62% for Acid Black 210. It was evident from the results that 1 h of the reaction time was sufficient for the maximum removal of dye (Table II). After 1 h of incubation time, a marginal amount of dye removal was noticed up to remaining 2 h of the incubation time. Acid Yellow 42 was decolorized up to 91% within 15 min of incubation time. Recently, Mohan et al. [23] demonstrated decolorization of Acid Black 10 BX by horseradish peroxidase (HRP), and dye decolorization was maximum in 45 min.

Decolorization of textile reactive dyes in the absence of HOBT was followed by the formation of precipitate, which settled down and could be removed by centrifugation. Several earlier investigators have shown that the treatment of phenols and aromatic amines by peroxidases and tyrosinases resulted in the formation of large insoluble aggregates [10,11]. However, the decolorization of acid dyes by TP in the presence of 2.0 mM HOBT appeared without the formation of any precipitate. It suggested that the decolorization of

Table III. Length of *A. cepa* roots and inhibition (%) by TP decolorized acid dyes.

Name of the dye	Test solution	Root length (cm)	% Inhibition
Acid Blue 92	Untreated	1.65	70.00
	Treated	0.85	84.54
Acid Red 97	Untreated	0.30	94.54
	Treated	1.30	76.36
Acid Yellow 42	Untreated	1.30	76.36
	Treated	1.90	65.45
Acid Black 1	Untreated	0.80	85.45
	Treated	0.90	83.36
Acid Black 210	Untreated	1.10	80.00
	Treated	0.82	85.09
	Control	5.50	–

Independent dye was treated as described in the text. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

dyes took place via degradation of aromatic ring of the compounds or by cleaving certain functional groups. These results were in agreement with earlier published findings [14,15,24]. Hence, HOBT could have a dual role, first as a mediator in increasing the substrate range of dyes for TP and second enhancing the rate of oxidation of various substrates.

Determination of phytotoxicity of decolorized product

In order to examine the toxicity of TP decolorized product of acid dyes, we performed phytotoxicity experiment by using *A. cepa* test with all the used dyes and their decolorized product. Table III shows the growth of *A. cepa* in terms of length in cm and percent inhibition brought about by dye solutions (untreated and treated). When *A. cepa* was incubated with dye solutions for 7 days, the maximum growth inhibition was recorded to be 94.54% for Acid Red 97 (untreated), the average root length was recorded to be 0.30 cm compared with 5.50 cm in control. The minimum inhibition in root length was 65.45% for TP-treated Acid Yellow 42 product as compared to control.

Table III also shows the effect of Acid Black 1 on the *A. cepa* root inhibition which further demonstrates the formation of non-toxic dye degraded products. However the degradation of Acid Blue 92 and Acid Black 210 brought about a growth inhibition of 84.54 and 85.09%, respectively, which is high in comparison with untreated dye solutions.

However, the product of some TP-treated acid dyes was more toxic as compared to parent dye. In order to get rid of toxic product, we investigated the decolorization of acid dyes in the presence of phenol and it produced insoluble product, which could be easily removed by centrifugation. Thus, it can fully minimize the risk of the product toxicity (data not given).

Effect of pH on the decolorization of dyes with TP

Five acid textile dyes were treated with TP in the buffers of different pH values (Figure 1). Most of the dyes were maximally decolorized at pH 5.0. As pH of the decolorizing sample was increased up to pH 10.0, the rate of decolorization decreased in all the treated dyes. Further, at higher pH Acid Red 97 and Acid Black 210 were marginally decolorized. However, Acid Blue 92, Acid Black 1 and Acid Yellow 42 showed no decolorization in

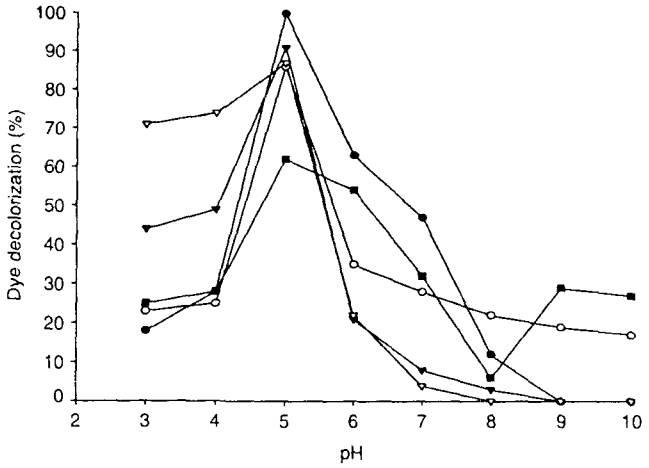

Figure 1. Effect of pH on the TP mediated decolorization of acid dyes. Each dye was treated with TP (0.235 U of TP mL^{-1}) in buffer of different pH value (3.0–10.0) at 37°C for 1 h. HOBT (2.0 mM) was used as a redox-mediator. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%. Symbols indicate (●) Acid Blue 92, (○) Acid Red 97, (▼) Acid Yellow 42, (▽) Acid Black 1 and (■) Acid Black 210.

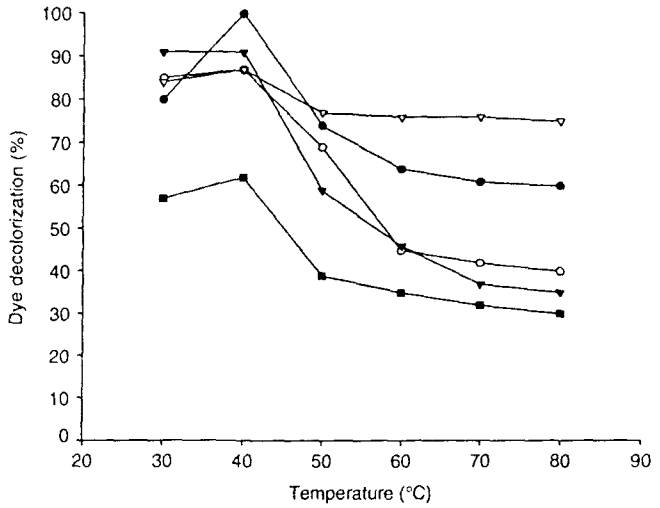

Figure 2. Effect of temperature on the TP mediated decolorization of acid dyes. Each dye was treated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 30–80°C for 1 h. HOBT (2.0 mM) was used as a redox-mediator. Each value represents the mean for three independent experiments performed in duplicate, with average SD <5%. For symbols refer to Figure 1.

alkaline range (Figure 1). In an earlier study it has been shown that HRP and bitter gourd peroxidase (BGP) could decolorize and degrade dyes maximally at pH 2.5 and 3.0 [14,25].

Effect of temperature on the decolorization of dyes with TP

Figure 2 shows the effect of different temperatures (30–80°C) on the decolorization of acid dyes. The decolorization of dyes was maximum at 40°C in case of all the tested dyes.

Table IV. Decolorization of polluted water containing mixture of dyes.

Mixture of acid dyes	λ_{max} (nm)	Decolorization (%) (+) HOBT	(−) HOBT
Acid Red 97 + Acid Yellow 42 + Acid Black 1 + Acid Blue 92	541	85	6
Acid Yellow 42 + Acid Black 1 + Acid Black 210 + Acid Blue 92	582	77	0
Acid Red 97 + Acid Black 210 + Acid Black 1 + Acid Blue 92	580	95	4
Acid Red 97 + Acid Yellow 42 + Acid Blue 92 + Acid Black 210	531	41	0
Acid Red 97 + Acid Yellow 42 + Acid Black 1 + Acid Black 210	439	37	0
Acid Red 97 + Acid Yellow 42 + Acid Black 1 + Acid Black 210 + Acid Blue 92	536	57	0

The mixtures of dyes were treated with TP (0.235 U mL^{-1}) in 100 mM sodium acetate buffer, pH 5.0 at 37°C for 1 h. HOBT (2.0 mM) was used as a redox-mediator for the selected experiments. In this table (−) and (+) signs indicate the decolorization of dyes in the absence and presence of HOBT, respectively. Each value represents the mean for three independent experiments performed in duplicate, with average standard deviation <5%.

Above and below this temperature rate of decolorization was decreased. These results were in agreement with earlier reported results; the decolorization of reactive dyes by BGP was maximum at 40°C [25].

Treatment of mixtures of dyes with TP

To understand the decolorization of dyes present in industrial effluent, we prepared complex mixtures of various acid dyes by mixing different dyes in equal proportions and incubated with 0.235 EU of TP mL^{-1} in the presence of 2.0 mM HOBT and 0.75 mM H_2O_2 for 1 h at 37°C (Table IV). The λ_{max} for each dye mixture was pre-determined and decolorization was monitored after incubation period at their λ_{max}. The mixtures were decolorized to a varying extent (37–95%) in the presence of 2.0 mM HOBT, whereas the complex mixtures were also recalcitrant to the TP action in the absence of redox-mediator.

Kinetics of acid dye decolorization

In order to determine the kinetic parameters of enzyme for various used dyes, an experiment with different dye concentrations, ranging from 15 to 200 mg L^{-1} was performed. The plot of initial rate versus dye concentration for all five acid dyes followed the hyperbolic pattern as expected for Michaelis–Menten kinetics (data not given). Moreover, the Lineweaver–Burk plot, inverse of initial rate versus inverse of substrate (dye) concentration is also found to be linear. The values of specific dye decolorization rate ($r_{dye\,max}$) estimated from the experimental data was 7.5 mg L^{-1} h^{-1} for Acid Blue 92, 79 mg L^{-1} h^{-1} for Acid Red 97, 6.5 mg L^{-1} h^{-1} for Acid Yellow 42, 4.2 mg L^{-1} h^{-1} for Acid Black 1 and 20.5 mg L^{-1} h^{-1} for Acid Black 210. The value of apparent Michaelis constant (K_m) was 89 mg L^{-1} for Acid Blue 92, 97.5 mg L^{-1} for Acid Red 97, 6.2 mg L^{-1} for Acid Yellow 42, 35.8 mg L^{-1} for Acid Black 1 and 16 mg L^{-1} for Acid Black 210 (Table V). The K_m value was lowest for the Acid Yellow 42, it showed the highest affinity of the enzyme for this dye. This is also evident from Table II that in the presence of HOBT the dye was decolorized 91% in 15 min. However, other dyes took more time to achieve the same decolorization.

Table V. Kinetics constants of TP for decolorization of dyes.

Name of the dye	$r_{dye\,max}$ (mg L^{-1} h^{-1})	K_m (mg L^{-1})
Acid Blue 92	7.5	89
Acid Red 97	79	97.5
Acid Yellow 42	6.5	6.2
Acid Black 1	4.2	35.8
Acid Black 210	20.5	16

Independent dye was treated as described in the text. Each value represents the mean for three independent experiments performed in duplicate, with average SD <5%.

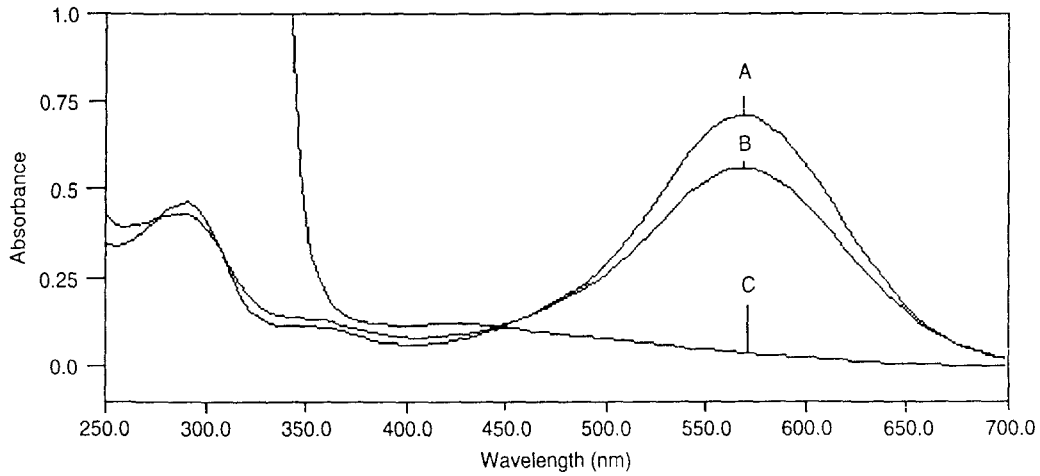

Figure 3. UV–Vis absorption spectra of Acid Blue 92. The dye was incubated with TP (0.235 U mL^{-1}) and 0.75 mM H_2O_2 in 100 mM sodium acetate buffer, pH 5.0 at 37°C for 1 h in the presence and absence of 2.0 mM HOBT. Spectra for the control and TP-treated dye solution were taken on Cintra 10*e* UV–Vis spectrophotometer. Untreated dye solution (A), treated dye solution in the absence of HOBT (B) and treated dye solution in the presence of HOBT (C).

UV–Vis spectral analysis

Figures 3 and 4 demonstrate the decolorization and degradation of Acid Blue 92 and a mixture of Acid Red 97, Acid Yellow 42, Acid Blue 92, and Acid Black 1, respectively by TP in the presence of 2.0 mM HOBT. These figures evidently demonstrate the rapid disappearance of absorption peaks in the visible region in the presence of HOBT. The disappearance of absorption peak in the presence of HOBT in visible region was due to the breakdown of chromophoric group present in the dyes [14]. The decolorization of Direct Fast Scarlet 4 BS in the microbial consortium composed of Pseudomonas 1–10 and White-rot fungus 8–4* also indicated the formation of intermediates with phenyl ring as the major components in the UV region, and its content was suggested to be more than in the original solution [26]. The absorbance peaks appeared in the UV region after TP treatment was quite comparable to the results explained by Fang et al. [26].

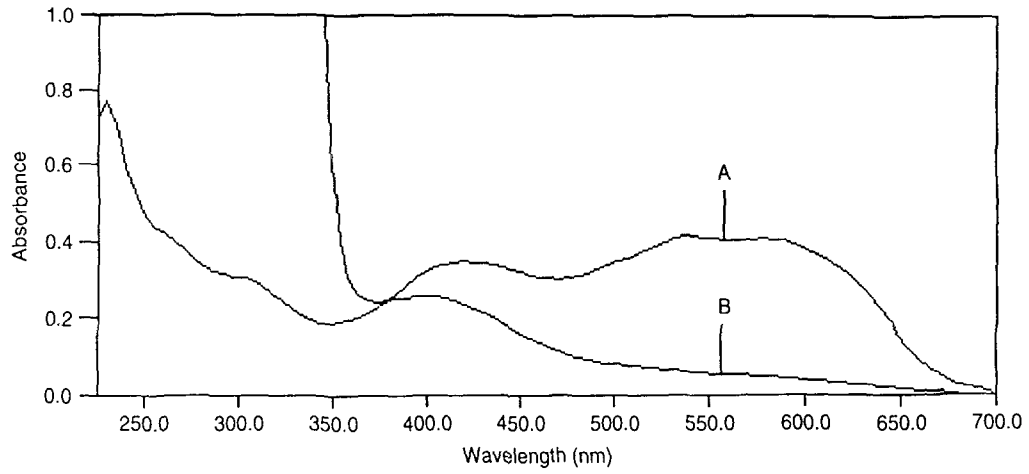

Figure 4. UV–Vis absorption spectra of a mixture of dyes. The dye mixture (Acid Red 97, Acid Yellow 42, Acid Blue 92 and Acid Black 1) was incubated with TP (0.235 U mL^{-1}) and 0.75 mM H_2O_2 in 100 mM sodium acetate buffer, pH 5.0 at 37°C for 1 h in presence of 2 mM HOBT. Spectra for the control and TP-treated dye mixture were taken on Cintra 10*e* UV–Vis spectrophotometer. Untreated dye mixture (A) and treated dye mixture in the presence of HOBT (B).

Bourbonnais and Paice [27] described for the first time the use of redox mediators by allowing laccase to oxidize non-phenolic compounds thereby expanding the range of substrates that can be oxidized by this enzyme. The mechanism of action of laccase mediator system has been extensively studied and it is used in textile industry in the finishing process for indigo stained materials. Several workers have demonstrated that use of enzyme/redox mediator system enhanced the rate of dye decolorization by several folds, but these mediators were required in very high concentrations (5.7 mM violuric acid/laccase system, 11.6 mM of HOBT/laccase system) [9,28]. In this study, for the first time, we have shown decolorization of dyes by TP by using very low concentration of HOBT (2.0 mM), which enhanced the rate of acid dyes decolorization by 5–92 folds. There were several reports about the enhancement of laccase activity by redox-mediators [9,28,29]. Most recently Akhtar et al. [24] have demonstrated the use of redox-mediator in the decolorization of dyes by using a peroxidase from bitter gourd. These results further support that peroxidases from other sources could also be used for the decolorization of recalcitrant dyes in the presence of redox mediators.

The potential application of TP for dye degradation was tested using a number of chemically diverse commercially available acid dyes. Dye solutions, were successfully treated with TP in the presence and absence of redox mediator, HOBT. Decolorization rate was drastically increased when industrially important acid dyes were treated with TP in the presence of 2.0 mM HOBT. In order to understand the application of TP in effluent treatment, we prepared complex mixtures of dyes. The complex mixtures of dyes treated with TP in the presence of HOBT were significantly decolorized. The application of peroxidase that is easily available and inexpensive can overcome its limitations in wastewater treatment. The use of peroxidases can be extended to the large-scale treatment of a wide spectrum of structural dye by using immobilized TP and relatively cheaper redox-mediators. This as well as the scale up of enzymatic processes is the subject of further study.

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